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(54) Title: USE OF POLYPEPTIDES OBTAINED THROUGH SYSTEMATIC MUTATIONS OF SINGLE AMINO ACIDS OF HUMAN AND NON-HUMAN BOX-A OF HMGB1 TO PREVENT AND/OR ANTAGONIZE PATHOLOGIES INDUCED BY HMGB1

(57) Abstract: The present invention relates to polypeptide variants of the HMGB-1 high affinity binding domain Box-A (HMGB1 Box-A) or to a biologically active fragment of HMGB1 Box-A, which are obtained through systematic mutations of single amino acids of the wild-type HMGB1 Box-A protein and which show an increased resistance to proteases and which are therefore characterized by more favourable pharmacokinetic and pharmacodynamic profiles. Moreover, the present invention concerns the use of said polypeptide molecules of HMGB1 Box-A to diagnose, prevent, alleviate and/or treat pathologies associated with extracellular HMGB1.

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Use of polypeptides obtained through systematic mutations of single amino acids of human and non-human Box-A of HMGB1 to prevent and/or antagonize pathologies induced by HMGB1

Description

The present invention relates to polypeptide variants of the HMGB-1 high affinity binding domain Box-A (HMGB1 Box-A) or to a biologically active fragment of HMGB1 Box-A, which are obtained through systematic mutations of single amino acids of the wild-type HMGB1 Box-A protein and which show an increased resistance to proteases and which are therefore characterized by more favourable pharmacokinetic and pharmacodynamic profiles. Moreover, the present invention concerns the use of said polypeptide molecules of HMGB1 Box-A to diagnose, prevent, alleviate and/or treat pathologies associated with extracellular HMGB1.

Recent research in the field of sepsis and inflammation has led to an improved understanding of the pathogenic mechanisms and events underlying their clinical onset and development. In the early stages of sepsis, for instance, bacterial endotoxins stimulate cells of the innate immune system which release pro-inflammatory cytokines (TNF, IL-1α and IL-6). These early cytokines in turn induce the release of a later-acting downstream mediator (identified as the known protein HMGB1) that triggers the pathological sequelae mediated by the subsequent release of cytokines such as TNF, IL-1α, IL-1β, IL-1Ra, IL-6, IL-8, IL-18, IFN-γ, PAF, etc., leading to a multisystem pathogenesis or to a lethal systemic inflammation (Andersson et al., 2002).

The HMGB1 protein belongs to the family of high mobility group (HMG) proteins. HMG proteins, so-called due to their high electrophoretic mobility in polyacrylamide gels, are the most ubiquitous non-histone proteins

associated with isolated chromatin in eukaryotic cells. These proteins play a generalized „architectural" role in DNA bending, looping, folding and wrapping, since they either distort, bend or modify DNA structures and complexes with transcription factors or histones (Andersson et al., 2002; Agresti et al., 2003; Degryse et al., 2003). The high mobility group 1 (HMGB1) protein is usually a nuclear factor, in particular a transcriptional regulatory molecule causing DNA bending and facilitating the binding of several transcriptional complexes.

Structurally, the HMGB1 protein is a protein of approximately 25 kDa with a highly conserved sequence among mammals, whereby 2 out of 214 amino acids have conservative substitutions in all mammalian species. HMGB1 is ubiquitously present in all vertebrate nuclei and in particular can be found in fibroblasts, neurons, hepatocytes, glia and in cells derived from hematopoietic stem cells, including monocytes/macrophages, neutrophils and platelets. The HMGB1 molecule has a tripartite structure composed of three distinct domains: two DNA binding domains called HMG Box-A and Box-B, and an acid carboxyl terminus, making it bipolarly charged.

The two HMGB1 boxes are involved in the protein's function as non-sequence-specific architectural DNA-binding elements, conferring the ability to bind DNA into recognized distorted DNA structures and stabilizing nucleosome assembly, remodelling and sliding. Both the A- and B-HMG boxes are made up of highly conserved 84 amino acid residues, are strongly positively charged and are arranged in three α-helices having a similar L-shaped fold. The long arm of the "L" contains the N-terminal extended strand and helix III (Andersson et al. 2002; Agresti et al., 2003; Thomas, J. O. 2001), while the short arm comprises helices I and II. Structure-function analysis reveals that the pro-inflammatory cytokine domain of HMGB1 is the B-Box and in particular the sequence of its first 20 amino acids. The A-Box is an extremely weak agonist of the inflammatory cytokine release triggered by HMGB1 and competitively inhibits the pro-inflammatory activities of the B-Box and of the whole protein. Therefore, from a pharmacological point of

view, the A-Box acts as an antagonist of the pathological conditions induced and/or sustained by the B-Box and HMGB1.

The third domain, the carboxyl terminus or acidic tail, is extremely negatively charged since it contains 30 repetitive aspartic and glutamic acid residues, and is linked to the boxes by a basic region of about 20 residues. Mouse and rat HMGB1 differ from the human form by only two substitutions that are located in this continuous C-terminal stretch.

HMGB1 binds rather weakly to the B-form variety of linear double-stranded DNA with no sequence specificity, while it binds in the interior of the nucleus with high affinity to supercoiled DNA, to unusual DNA structures like 4-way junctions (cruciform DNA), bulged DNA and bent DNA (Ferrari et al., 1992; Pontiggia et al., 1993 and PCT/EP2005/007198 in the name of Creabilis Therapeutics).

Besides its nuclear location and role as a transcription factor regulator, HMGB1 has also been found in the extracellular medium, actively released by activated cells of the immune systems (monocytes and macrophages) or passively released by damaged or necrotic cells (Andersson et al., 2002; Scaffidi et al., 2002; Bonaldi et a., 2002; Taniguchi et al., 2003; Friedman et al., 2003; Palumbo et al., 2004).

Extracellularly released HMGB1 acts as a potent cytokine and as an extremely potent macrophage-stimulating factor. HMGB1 acts directly by binding to the cell membrane, inducing signaling and chemotaxis, having a chemokine-like function (Yang et al., 2001) and further acting indirectly by up-regulating the expression and secretion of pro-inflammatory cytokines. This makes extracellular HMGB1 protein a potent chemotactic and immunoregulatory protein which promotes an effective inflammatory immune response. Furthermore, other proteins belonging to the family of HMG proteins, and which are able to bend DNA, are released together with HMGB1 in the extracellular medium. These proteins are inter alia HMGB2,

HMGB3, HMG-1L10, HMG-4L and SP100-HMG. They share with HMGB1 highly homologous amino acid sequences. Like HMGB1, they trigger/sustain inflammatory pathologies interacting with the same receptors, leading to the same downstream pathways of interaction.

In healthy cells, HMGB1 migrates to the cytoplasm both by passive and active transport. However, all cultured cells and resting monocytes contain the vast majority of HMGB1 in the nucleus, indicating that in baseline conditions import is much more effective than export. Cells might transport HMGB1 from the nucleus by acetylating lysine residues which are abundant in HMGB1, thereby neutralizing their basic charge and rendering them unable to function as nuclear localization signals. Nuclear HMGB1 hyperacetylation determines the relocation of this protein from the nucleus to the cytoplasm (in the fibroblasts, for example) or its accumulation into secretory endolysosomes (in activated monocytes and macrophages, for example) and subsequent redirection towards release through a non-classical vesicle-mediated secretory pathway. HMGB1 secretion by already activated monocytes is then triggered by bioactive lysophosphatidylcholine (LPC), which is generated later in the inflammation site from phosphatidylcholine through the action of the secretory phospholipase sPLA2 produced by monocytes several hours after activation. Therefore, secretion of HMGB1 seems to be induced by two signals (Bonaldi et al., 2003) and to take place in three steps: 1) at first, an inflammatory signal promotes HMGB1 acetylation and its relocation from the nucleus to the cytoplasm (step 1) and storage in cytoplasmic secretory vesicles (step 2); then, a secretion signal (extracellular ATP or lysophosphatidylcholine) promotes exocytosis (third step) (Andersson et al., 2002; Scaffidi et al. 2002; Gardella et al., 2002; Bonaldi et al., 2003; Friedman et al., 2003).

Released HMGB1 has been identified as one of the ligands binding to the RAGE receptor. This receptor is expressed in most cell types, and at a high level mainly in endothelial cells, in vascular smooth muscle cells, in

monocytes and macrophages and in mononuclear phagocytes. Recognition involves the C-terminal of HMGB1. The interaction of HMGB1 and RAGE triggers a sustained period of cellular activation mediated by RAGE up-regulation and receptor-dependent signaling. In particular, the interaction of HMGB1 and RAGE activates several intracellular signal transduction pathways, including mitogen-activated protein kinases (MAPKs), Cdc-42, p21ras, Rac and the nuclear translocation factor κB (NF-κB), the transcription factor classically linked to inflammatory processes (Schmidt et al., 2001).

According to several experimental evidences, released HMGB1 may also interact with receptors belonging to one or more subclasse(s) of the family of the Toll-like receptors. Further, HMGB1 may also interact with the functional N-terminal lectin-like domain (D1) of thrombomodulin. Due to the ability of the functional D1 domain of thrombomodulin to intercept and bind circulating HMGB1, the interaction with the RAGE receptors and the Toll-like receptors is prevented.

In the context of the present invention, "HMGB1" includes the non-acetylated form or/and the acetylated form of HMGB1. Likewise, "HMGB1 homologous proteins" include the non-acetylated form or/and the acetylated form of HMGB1 homologous proteins. Preferred HMGB1 homologous proteins are HMGB2, HMGB3, HMG-1L10, HMG-4L or/and SP100-HMG.

When released *in vivo*, HMGB1 is an extremely potent cytokine and a potent macrophage-stimulating factor. In fact, like other cytokine mediators of endotoxemia, HMGB1 activates *in vitro* a cascade of multiple pro-inflammatory cytokines (TNF, IL-1α, IL-1β, IL-1Ra, IL-6, IL-8, MIP-1α and MIP-1β) from human macrophages. Therefore, HMGB1 acts as a late mediator during acute inflammation and participates in an important way in the pathogenesis of systemic inflammation after the early mediator response has been resolved.

The observed pro-inflammatory effects of HMGB1 *in vitro* and the correlation between circulating HMGB1 levels and the development of the pathogenic sequence of systemic inflammation *in vivo* indicate that therapeutically targeting of this cytokine-like molecule should be of relevant clinical value, suggesting novel therapeutic approaches by a "late" administration of (selective) antagonists/ inhibitors of the extracellular activities of HMGB1.

Therefore, several attempts were performed in order to block this extracellular HMGB1 chemo-cytokine protein. Several important approaches were addressed to the administration of antibodies against HMGB1, of HMGB1 fragments (for example HMGB1 A-Box), of antibodies to RAGE, of soluble RAGE (sRAGE) and of ethyl pyruvate (Czura et al., 2003; Lotze et al., 2003).

The passive immunization of mice with HMGB1-neutralizing antibodies conferred a highly significant, dose-dependent and lasting protection against lethal doses of endotoxin, even when the first doses of antibodies were given after the TNF peak had passed, suggesting that antagonizing HMGB1 activity late in the clinical course may be an effective treatment approach to potentially lethal sepsis (Yang et al., 2004).

Another possibility is to administer mono- or oligoclonal antibodies against the HMGB1 B-Box, or its 20 amino acid relevant core which signals through RAGE. Furthermore, HMGB1 A-Box, one of the two DNA-binding domains in HMGB1, has been identified as a specific antagonist of HMGB1: highly purified recombinant A-Box has protected mice from lethal experimental sepsis even when initial treatment has been delayed for 24 hours after pathology induction, further suggesting that HMGB1 antagonists may be administered successfully in a clinically relevant window wider than the one used for other known cytokines (Yang et al., 2004).

Structural function analysis of HMGB1-truncated mutants has revealed that the A-Box domain of HMGB1 competitively displaces the saturable binding of HMGB1 to macrophages, specifically antagonizing HMGB1 activities. As has been already seen with the protective activity of anti-HMGB1 antibodies, the administration of the A-Box rescues mice from sepsis even when treatment has been initiated as late as 24 hours after surgical induction of sepsis (Yang H. et al., 2004). HMGB1 antagonists or inhibitors selected from the group of antibodies or antibody fragments that bind to an HMGB1 protein, HMGB1 gene antisense sequences and HMGB1 receptor antagonists are known from US 6,468,533, WO 02/074337 and US 2003/0144201.

Moreover, saturation of circulating HMGB1 by the administration of sRAGE leads to the block of its activities mediated by cellular RAGE, a result which can also be obtained by inhibiting RAGE itself with the administration of anti-RAGE antibodies.

Furthermore, a similar protective response late in the course of sepsis has been observed by administering ethyl-pyruvate, a stable lipophilic derivative and relatively non-toxic food additive also used as an experimental anti-inflammatory agent, which attenuates the systemic inflammation of ischemia/reperfusion tissue injury and lethal hemorrhagic shock. Ethyl-pyruvate inhibited HMGB1 and TNF release *in vitro* from endotoxin-stimulated murine macrophages, while *in vivo* protected mice from peritonitis-induced lethal sepsis, again when dosing was begun 24 hours after this pathology was experimentally induced.

Finally, it has been shown that the N-terminal lectin-like domain (D1) of thrombomodulin is an inhibitor of HMGB1, since it binds to and sequesters this chemokine, preventing the binding of HMGB1 to RAGE and Toll-like

receptors such that the downstream cascade of events leading to inflammatory pathologies is inhibited.

As described above, several attempts were performed with the aim of inhibiting and/or antagonising the extracellular HMGB1 chemo-cytokine protein. The present invention is based on the experimental evidence that the two high affinity binding domains for DNA, i.e. HMGB1 Box-A and HMGB1 Box-B, which are present in the HMGB1 molecule, have two opposing roles in the protein released in the extracellular space. The main activity of HMGB1 Box-A is to mediate the pro-inflammatory activities attributed to the HMGB1 protein. On the other hand, HMGB1 Box-A acts as an antagonist competing with the pro-inflammatory activity of the Box-B domain.

The problem underlying the present invention was therefore the provision of novel agents for the prevention, alleviation and/or treatment of HMGB1-associated pathologies. In particular, the problem of the present invention was to develop novel agents as selective extracellular HMGB1 antagonist and/or inhibitors, in order to prevent, alleviate and/or treat the broad spectrum of pathological effects induced by the HMGB1 chemokine itself and/or by the cascade of multiple inflammatory cytokines caused by the extracellular release of the HMGB1 protein.

The solution to the above problem is therefore the provision of a polypeptide variant of the human and/or non-human HMGB1 high affinity binding domain Box-A (HMGB1 Box-A) or of a biologically active fragment of human and/or non-human HMGB1 Box-A, characterized in that the amino acid sequence of said polypeptide variant differs from the amino acid sequence of the wild type HMGB1 Box-A protein by the mutation of one or more single amino acids. Surprisingly, it was found by the inventors of the present invention that said polypeptide variant exhibits an increased resistance to proteolysis

compared to wild type HMGB1 Box-A or to the biological active fragment of the wild type HMGB1 Box-A.

By increasing the resistance to the proteolytic activity of the proteases, a more favourable pharmacokinetic and pharmacodynamic profile can be achieved, since an increased stability in body fluids is obtained for the inventive polypeptide variants. As a result thereof, an increase in the half-life in body fluids of the protein's variants of the present invention is observed as well. It is known that the estimated half-life of proteins *in vivo* can be as short as a few minutes. The variants of the present invention preferably have an increased half-life, e.g. because they are more resistant to proteases.

In a most preferred embodiment of the present invention, polypeptide variants are obtained by using a directed evolution process, which technology is extensively described in WO 2004/7022593 and in several further patent applications (PCT/FR00/03503, PCT/FR01/01366, US 10/022,249, US 10/022,390, US 10/375,192, US 60/409,898, US 60/457,135, US 60/410,258 and US 60/410,263), all in the name of Nautilus Biotech S.A. (Paris, France), which are herein incorporated by reference.

In general, the term "directed evolution" refers to biotechnological processes devoted to the improvement of target protein features by means of specific changes introduced into their amino acid sequences. The directed evolution process includes the generation of a library of mutant versions of the gene of interest, followed by the selection of those variants that display the desired features. These processes can be iterative when gene products having an improvement in a desired property are subjected to further cycles of mutation and screening.

In order to optimise the Box-A of HMGB1 protein and to obtain the polypeptide variants of the present invention with higher stability against

proteases, a particular Nautilus proprietary technology for directed evolution has been applied. In particular, a so-called two-dimensional rational mutagenesis scanning approach ("2-D scanning") has been applied, which is described in the Nautilus patent application WO 2004/022593, said application being herein incorporated by reference.

Nautilus 2-D scanning approach for protein rational evolution is based on a process, in which two dimensions of the target protein are scanned by serial mutagenesis in order to find the right amino acid change that is needed at the right amino acid position. The first dimension that is scanned is the amino acid position along the target protein sequence, in order to identify those specific amino acid residues to be replaced with different amino acids. These amino acid positions are referred to as is-HIT target positions. The second dimension is the specific amino acid type selected for replacing a particular is-HIT target position. According to a particular approach of the 2-D scanning method, a number of target positions along the protein sequence are selected, *in silico.* As used herein, *in silico* refers to research and experiments performed using a computer. In this context, *in silico* methods include, but are not limited to, molecular modeling studies and biomolecular docking experiments. Therefore, the amino acid target positions on the protein sequence are identified without use of experimental biological methods. Once a protein feature to be optimised is selected, diverse sources of information or previous knowledge are exploited in order to determine those amino acid positions that may be amenable to improve the protein's fitness by replacement with a different amino acid. In particular the "is-HIT target positions" are identified based on three factors, being (i) the protein feature to be evolved and optimised, (ii) the protein's amino acid sequence and/or (iii) the known properties of the individual amino acids.

In the specific context of the present invention, the "*in silico* HITs" ("is-HITs") are all possible candidate amino acid positions along the target protein's primary sequence that might be involved as target for the proteolytic activity of proteases. Based on the specific list of proteases considered in the

context of the present invention (Fig. 1), the complete list of all amino acid sequences that could potentially be targeted within the wild type HMGB1 Box-A amino acid sequence is determined.

Once the is-HIT target positions have been selected, mutagenesis then is performed by the replacement of single amino acid residues at the specific acid target positions on the protein backbone. The mutagenesis is performed by residue replacement "one-by-one" in addressable arrays and molecules containing the preselected amino acid changes at each of the target amino acid positions are produced.

The choice of the replacing amino acid takes into account the need to preserve the physicochemical properties such as hydrophobicity, charge and/or polarity of essential residues (such as catalytic and binding residues). Numerous methods of selecting replacing amino acids are well known in the art, in particular, amino acid substitution matrixes are used for this purpose. A very preferred technology according to the present invention makes use of the so-called "Percent Accepted Mutation" (PAM) (Dayhoff et al., Atlas of protein sequence and structure, 5(3):345-352, 1978), as shown in Fig. 2. PAM values are used in order to select an appropriate group of replacement amino acids. PAM values, originally developed to produce alignments between protein sequences, are available in the form of probability matrixes, which reflect an evolutionary distance. "Conservative substitutions" of a residue in a reference sequence are those substitutions that are physically and functionally similar to the corresponding reference residues, e.g. those that have a similar size, shape, electric charge, chemical properties, including the ability to form covalent or hydrogen bonds, or the like. Preferred conservative substitutions show the highest scores fitting with the PAM matrix criteria in the form of "accepted point mutations". The PAM250 matrix is used in 2-D scanning to identify the replacing amino acids for the is-HITs in order to generate conservative mutations without affecting the protein function. At least, the two amino acids with the highest values in PAM250 matrix, corresponding to "conservative substitutions" or "accepted

point mutations", are chosen. The replacement of amino acids by cysteine residues is explicitly avoided, since this change would potentially lead to the formation of intermolecular disulfide bonds.

Using the above-resumed Nautilus Biotech directed evolution technology, the inventors of the present application were able to obtain polypeptide variants of the HMGB1 Box-A which differ from the amino acid sequence of the native target polypeptide by one or more mutation.

In the context of the present invention, where reference is made to the term "HMGB1 Box-A or amino acid sequence of HMGB1 Box-A", it is referred to both human and non-human HMGB1 Box-A. In a preferred embodiment of the present invention, the systematic mutation of single amino acid on the critical is-HITs positions for proteases has been obtained on the wild type of human HMGB1 Box-A protein and on the wild type of *Anopheles gambia* HMGB1 Box-A protein. The choice of the species *Anopheles gambia* was made by the inventors of the present application after a proper structural and phylogenetic analysis showing a 68% identity and a 88% homology of the human and *Anopheles HMGB1* Box-A.

"Biologically active fragments of HMGB1 Box-A" as used herein are meant to encompass parts of the known wild type HMGB1 Box-A protein, for which at least one of the biological activities of the corresponding mature protein is still observable when known tests are being used. Preferably, a fragment of the mature protein is considered as biologically active if an antagonist activity with respect to the pro-inflammatory activity of the HMGB1 B-Box and the HMGB1 protein as a whole can be determined. Biologically active fragments of native HMGB1 Box-A are fragments of at least 20, 25, 30, 35, 45, 50, 55, 60, 65, 70, 75 or 80 amino acids. Preferred biologically active fragments of native HMGB1 Box-A used in the context of the present invention comprises fragments of at least 77 or of at least 54 amino acids, respectively.

The term "mutation" as used in the context of the present invention can be understood as substitution, deletion and/ or addition of single amino acid in the target sequence. Preferably, the mutation of the target sequence in the present invention is a substitution. The substitution can occur with different genetically encoded amino acid or by non-genetically encoded amino acids. Examples for non-genetically encoded amino acids are homocystein, hydroxyproline, ornithin, hydroxylysine, citrulline, carnitine, etc.

The polypeptide variants of the present invention obtained by using directed evolution technology are mutant proteins which differ from the amino acid sequence of the wild type HMGB1 Box-A by the mutation of one or more single amino acid. In a very preferred embodiment of the present invention, only one amino acid replacement occurs on the sequence of the native protein. In this case, the polypeptide variant of the invention is obtained by the modification of the native protein, such that the amino acid sequence of the variant differs from that of the native protein by a single amino acid change at only one of the is-HIT target positions. It is, however, encompassed by the subject of the present invention that the native protein can be further optimised by replacement of a plurality, e.g two or more, of is-HIT target positions on the same protein molecule. According to this variant of the invention, polypeptide variants are obtained by combining the single mutation into a single protein molecule. The modified polypeptide variants having more single amino acid replacement can differ from the wild type protein sequence by amino acid replacements on 1-10, preferably 2, 3, 4, 5 and 6 different amino acid target positions.

The selection of the candidate lead of the series of polypeptide variants produced with the technology used in the present invention is based both on the more favourable pharmacokinetic profile, obtained thanks to the longer resistance to proteases and on a better pharmacodynamic profile thanks to an increased intrinsic activity and binding affinity which gives a greater antagonistic activity than the native HMGB1 Box-A protein.

In a particular embodiment of the invention, starting from Human HMGB1 Box-A as starting native protein, three groups of polypeptide variants are obtained. In particular, one group of polypeptide variants is derived from single mutations introduced into the full-length amino acid sequence (84 amino acids) from Human HMGB1 Box-A. The other two groups of inventive polypeptide variants are generated starting from biologically active fragments of Human HMGB1 Box-A of 77 amino acids and 54 amino acids, respectively.

In a further particular embodiment of the invention, starting from *Anopheles gambia* HMGB1 Box-A as starting native protein, three groups of polypeptide variants are obtained. In particular, one group of polypeptide variants is derived from single mutations introduced into the full-length amino acid sequence (84 amino acids) from *Anopheles gambia* HMGB1 Box-A. The other two groups of inventive polypeptide variants are generated starting from biologically active fragments of *Anopheles gambia* HMGB1 Box-A of 77 amino acids and 54 amino acids, respectively.

Hence, the above-mentioned very preferred polypeptide variants of this invention are defined as below.

1) On the human HMGB1 Box-A full-length fragment of 84 amino acids defined by the sequence SEQ ID NO:1 (Fig. 3a), 53 amino acid positions, recognized as substrate for different proteases (cf. Fig. 1), are identified. The numbering corresponds to that in the wild type protein:

K2, D4, P5, K6, K7, P8, R9, K11, M12, Y15, F17, F18, R23, E24, E25, K27, K28, K29, P31, D32, F37, E39, F40, K42, K43, E46, R47, W48, K49, M51, K54, E55, K56, K58, F59, E60, D61, M62, K64, D66, K67, R69, Y70, E71, R72, E73, M74, K75, Y77, P79, P80, K81, E83.

The native amino acid at each of these positions is replaced by residues defined by the susbtitution matrix PAM250 (cf. Fig. 2). In particular, the

performed residue substitutions are as listed below.

R to H, Q
E to H, Q, N
K to Q, T
D to N, Q
M to I, V
P to A, S
Y to I, H
F to I, V
W to Y, S

A total of 115 polypeptide variants of Box-A of human HMGB1 are generated (Fig. 3a). These polypeptide variants are defined in sequences SEQ ID NOs:2 to 116.

2) On the Human HMGB1 Box-A biologically active fragment of 77 amino acids, defined in sequence SEQ ID NO:117 (Fig. 4a), 48 amino acid positions, recognized as substrate for different proteases (cf. Fig. 1), are identified. The numbering is in accordance to their position in SEQ ID NO:117:

P1, R2, K4, M5, Y8, F10, F11, R16, E17, E18, K20, K21, K22, P24, D25, F30, E32, F33, K35, K36, E39, R40, W41, K42, M44, K47, E48, K49, K51, F52, E53, D54, M55, K56, D59, K60, R62, Y63, E64, R65, E66, M67, K68, Y70, P72, P73, K74, E76.

The native amino acid in each of these positions is replaced by residues defined by the susbtitution matrix PAM250 (cf. Fig. 2). In particular, the performed residue substitutions are as listed below.

R to H, Q
E to H, Q, N

K to Q, T
D to N, Q
M to I, V
P to A, S
Y to I, H
F to I, V
W to Y, S

A total of 105 polypeptide variants of Box-A of human HMGB1 fragment of 77 amino acids are generated (Fig. 4b) and defined as in sequences SEQ ID NOs:118 to 222.

3) On the Human HMGB1 Box-A biologically active fragment of 54 amino acids defined in sequence SEQ ID NO:223 (Fig. 5a), 35 amino acid positions, recognized as substrate for different proteases (Fig. 1), are identified. The numbering is in accordance to their position in SEQ ID NO:223:

P1, D2, F7, E9, F10, K12, K13, E16, R17, W18, K19, M21, K24, E25, K26, K28, F29, E30, D31, M32, K34, D36, K37, R39, Y40, E41, R42, E43, M44, K45, Y47, P49, P50, K51, E53.

The native amino acid at each of these positions is replaced by residues defined by the substitution matrix PAM250 (cf. Fig. 2). In particular, the performed residue substitutions are as listed below.

R to H, Q
E to H, Q, N
K to Q, T
D to N, Q
M to I, V
P to A, S
Y to I, H

F to I, V
W to Y, S

A total of 77 polypeptide variants of Box-A of human HMGB1 fragment of 54 amino acids are generated (Fig. 5b) and defined as in sequences SEQ ID NOs:224 to 300.

4) On the *Anopheles gambia* (XP_311154) HMGB1 Box-A full-length fragment of 84 amino acids, defined by the sequence SEQ ID NO:301 (Fig. 6a), 53 amino acid positions, recognized as substrate for different proteases (Fig. 1), were identified. The numbering is in accordance with the position in the native protein.

K2, K4, D5, K7, P8, R9, R11, M12, Y15, F17, F18, R23, E24, E25, K27, K28, K29, P31, E32, E33, F37, E39, F40, R42, K43, E46, R47, W48, K49, M51, L52, D53, K54, E55, K56, R58, F59, E61, M62, E64, K65, D66, K67, R69, Y70, E71, L72, E73, M74, Y77, P79, P80, K81.

The native amino acid at each of these positions was replaced by residues defined by the susbtitution matrix PAM250 (cf. Fig. 2).
The performed actual residue substitutions are as listed below.

R to H, Q
E to H, Q, N
K to Q, T
D to N, Q
M to I, V
P to A, S
Y to I, H
F to I, V
W to Y, S

A total of 117 variants of Box A of HMGB1 *Anopheles gambia* (XP_311154)

were generated (Fig. 6b) and identified in the sequences as defined in SEQ ID NOs:302 to 418.

5) On the *Anopheles gambia* (XP_311154) HMGB1 Box-A biologically active fragment of 77 amino acids, defined in sequence SEQ ID NO:419 (Fig. 7a), 49 amino acid positions, recognized as substrate for different proteases (cf. Fig. 1), were identified. The numbering is in accordance with the position in the sequence as defined in SEQ ID NO:419.

P1, R2, R4, M5, Y8, F10, F11, R16, E17, E18, K20, K21, K22, P24, E25, E26, F30, E32, F33, R35, K36, E39, R40, W41, K42, M44, L45, D46, K47, E48, K49, R51, F52, E54, M55, E57, K58, D59, K60, R62, Y63, E64, L65, E66, M67, Y70, P72, P73, K74.

The native amino acid at each of these positions was replaced by residues defined by the susbtitution matrix PAM250 (cf. Fig. 2).
The performed actual residue substitutions are as listed below.

R to H, Q
E to H, Q, N
K to Q, T
D to N, Q
M to I, V
P to A, S
Y to I, H
F to I, V
W to Y, S

A total of 109 polypeptide variants of Box-A of HMGB1 fragment of 77 amino acids were generated (Fig. 7b) and identified as defined in sequences SEQ ID NOs:420 to 529.

6) On the *Anopheles gambia* (XP_311154) HMGB1 Box-A biologically active

fragment of 54 amino acids defined in sequence SEQ ID NO:530 (Fig. 8a), 36 amino acid positions, recognized as substrate for different proteases (cf. Fig. 1), were identified. The numbering is in accordance with the position on the sequence as defined in SEQ ID NO:530.

P1, E2, E3, F7, E9, F10, R12, K13, E16, R17, W18, K19, M21, L22, D23, K24, E25, K26, R28, F29, E31, M32, E34, K35, D36, K37, R39, Y40, E41, L42, E43, M44,Y47, P49, P50, K51.

The native amino acid in each of these positions was replaced by residues defined by the substitution matrix PAM250 (cf. Fig. 2).
The performed actual residue substitutions are as listed below.

R to H, Q
E to H, Q, N
K to Q, T
D to N, Q
M to I, V
P to A, S
Y to I, H
F to I, V
W to Y, S

A total of 81 polypeptide variants of Box-A of HMGB1 *Anopheles gambia* (XP_311154) fragment of 54 amino acids were generated (Fig. 8b) and identified in the sequences as defined in SEQ ID NOs:531 to 612.

It is noted that the amino acids which occur in the various amino acid sequences appearing herein are identified according to their known one-letter code abbreviations. It should be further noted that all amino acid residue sequences represented herein by their one-letter abbreviation code have a left-to-right orientation in the conventional direction of amino-terminus to carboxyl-terminus.

Accordingly, the present invention provides modified polypeptide variants that exhibit increased resistance to the proteolytic activity of proteases and/or peptidases compared to the wild type HMGB1 Box-A protein. The polypeptide variants of the invention in particular exhibit an increase in the resistance to the proteolytic activity of the human proteases and/or peptidases, in particular of the human serum proteases and/or human gastro-intestinal proteases or peptidases. Preferred proteases are listed in Fig. 1. In a more preferred embodiment of the invention, polypeptide variants exhibit an increase in the resistance to the proteolytic activity of at least a protease selected from the group comprising chymotrypsin, trypsin, endoprotease, endopeptidases or a combination thereof.

In particular, the resistance to proteolysis is at least 10%, 20%, 30%, 40%, 50%, 70%, 80%, 90%, 95% or higher compared to the unmodified wild type HMGB1 Box-A. Protease resistance was measured at different timepoints (between 5 minutes and 8 hours) at 25°C after incubation of 20 µg of Box-A wild type or variants with a mixture of proteases at 1% w/w of total proteins. The mixture of the proteases was prepared freshly at each assay from stock solutions of endoproteinase Glu-C (SIGMA) 200 µg/ml; trypsin (SIGMA) 400µg/ml and α-chymotrypsin (SIGMA) 400 µg/ml. After protease incubation the reaction was stopped adding 10 µl of anti-proteases solution (Roche) and the samples were stored at –20°C for the biological activity assay.

As a consequence of the increased stability due to the increased resistance to proteases activity, the polypeptide variants of the present invention also exhibit a longer half-life in body fluids compared to the wild type HMGB1 Box-A. In particular, the half-life in serum and/or in blood is increased, whereby an increase of at least 10 minutes, 20 minutes, 30 minutes, 60 minutes or even longer, compared to the wild type HMGB1 Box-A is observed.

A further aspect of the present invention is a nucleic acid molecule encoding

a polypeptide variant of the present invention. In particular, the present invention refers to nucleic acid molecules encoding polyeptide variants as defined in SEQ ID NO:2 to 116, 118 to 222, 224 to 300, 302 to 418, 420 to 526 and 531 to 612.

A still further aspect of the present invention is a vector comprising a nucleic acid molecule as defined above.

Furthermore, the present invention refers to a method for producing a polypeptide variant as described above comprising (i) introducing a nucleic acid molecule as defined above into a host cell and (ii) culturing the cell, under conditions in which the encoded polypeptide variant is expressed. Preferably the host cell is a mammalian, insect or bacterial cell, in particular E. Coli, preferably the M15 strain.

A further method for producing a polypeptide variant as described above is the use of chemical peptide synthesis, e.g. a solid phase peptide synthesis according to standard methods.

The polypeptide variants of the present invention exhibit an increased resistance to proteolysis and thus a higher stability compared to the unmodified wild type protein. Consequently, the peptides of the invention also exhibit improved therapeutic and biological properties and activity. In fact, they show a more favorable pharmacokinetic and pharmacodynamic profile than native HMGB1 Box-A.

The invention is therefore directed to the use of the above-mentioned polypeptide variants of HMGB1 Box-A, obtained through systematic mutations of single amino acids in the sequence of HMGB1 Box-A or of its biologically active fragments as active agent in a medicament.

A still further aspect of the invention is hence the use of the inventive polypeptide variants for the manufacture of a medicament for the prevention

and/or treatment of extracellular HMGB1-associated pathologies or pathologies associated with the HMGB1 homologous proteins. In particular, the HMGB1 associated pathologies are pathologies which are mediated by a multiple inflammatory cytokine cascade.

The broad spectrum of pathological conditions induced by the HMGB1-chemokine and by the HMGB1-induced cascade of inflammatory cytokines are grouped in the following categories: inflammatory disease, autoimmune disease, systemic inflammatory response syndrome, reperfusion injury after organ transplantation, cardiovascular affections, obstetric and gynecologic disease, infectious (viral and bacterial) disease, allergic and atopic disease, solid and liquid tumor pathologies, transplant rejection diseases, congenital diseases, dermatological diseases, neurological diseases, cachexia, renal diseases, iatrogenic intoxication conditions, metabolic and iodiopathic diseases.

HMGB1-associated pathologies according to the present invention are preferably pathological conditions mediated by activation of the inflammatory cytokine cascade. Non limiting examples of conditions which can be usefully treated using the present invention include the broad spectrum of pathological conditions induced by the HMGB1-chemokine and by the HMGB1-induced cascade of inflammatory cytokines grouped in the following categories: restenosis and other cardiovascular diseases, reperfusion injury, inflammation diseases such as inflammatory bowel disease, systemic inflammation response syndrome, e.g. sepsis, adult respiratory distress syndrome, etc, autoimmune diseases such as rheumatoid arthritis and osteoarthritis, obstetric and gynaecological diseases, infectious diseases, atopic diseases, such as asthma, eczema, etc, tumor pathologies, e.g. solid or non-solid tumor diseases associated with organ or tissue transplants, such as reperfusion injuries after organ transplantation, organ rejection and graft-versus-host disease, congenital diseases, dermatological diseases such as psoriasis or alopecia, neurological diseases, ophthalmological diseases, renal, metabolic or idiopathic diseases and intoxication conditions,

e.g. iatrogenic toxicity, wherein the above diseases are caused by, associated with and/or accompanied by HMGB1 protein release.

In particular, the pathologies belonging to inflammatory and autoimmune diseases include rheumatoid arthritis/seronegative arthropathies, osteoarthritis, inflammatory bowel disease, Crohn's disease, intestinal infarction, systemic lupus erythematosus, iridoeyelitis/uveitis, optic neuritis, idiopathic pulmonary fibrosis, systemic vasculitis/Wegener's granulomatosis, sarcoidosis, orchitis/vasectomy reversal procedures. Systemic inflammatory response includes sepsis syndrome (including gram positive sepsis, gram negative sepsis, culture negative sepsis, fungal sepsis, neutropenic fever, urosepsis, septic conjunctivitis), meningococcemia, trauma hemorrhage, hums, ionizing radiation exposure, acute and chronic prostatitis, acute and chronic pancreatitis, appendicitis, peptic, gastric and duodenal ulcers, peritonitis, ulcerative, pseudomembranous, acute and ischemic cholitis, diverticulitis, achalasia, cholangitis, cholecystitis, enteritis, adult respiratory distress syndrome (ARDS). Reperfusion injury includes post-pump syndrome and ischemia-reperfusion injury. Cardiovascular disease includes cardiac stun syndrome, myocardial infarction and ischemia, atherosclerosis, thrombophlebitis, endocarditis, pericarditis, congestive heart failure and restenosis. Obstetric and gynecologic diseases include premature labour, endometriosis, miscarriage, vaginitis and infertility. Infectious diseases include HIV infection/HIV neuropathy, meningitis, B- and C-hepatitis, herpes simplex infection, septic arthritis, peritonitis, E. coli 0157:H7, pneumonia epiglottitis, haemolytic uremic syndrome/thrombolytic thrombocytopenic purpura, candidiasis, filariasis, amebiasis, malaria, Dengue hemorrhagic fever, leishmaniasis, leprosy, toxic shock syndrome, streptococcal myositis, gas gangrene, mycobacterium tuberculosis, mycobacterium avium intracellulare, pneumocystis carinii pneumonia, pelvic inflammatory disease, orchitis/epidydimitis, legionella, Lyme disease, influenza A, Epstein-Barr Virus, Cytomegalovirus, viral associated hemiaphagocytic syndrome, viral encephalitis/aseptic meningitis. Allergic and atopic disease include asthma, allergy, anaphylactic shock, immune complex disease, hay fever, allergic

rhinitis, eczema, allergic contact dermatitis, allergic conjunctivitis, hypersensitivity pneumonitis. Malignancies (liquid and solid tumor pathologies) include ALL, AML, CML, CLL, Hodgkin's disease, non Hodgkin's lymphoma, Kaposi's sarcoma, colorectal carcinoma, nasopharyngeal carcinoma, malignant histiocytosis and paraneoplastic syndrome/hypercalcemia of malignancy. Transplant diseases include organ transplant rejection and graft-versus-host disease. Congenital disease includes cystic fibrosis, familial hematophagocytic lymphohistiocytosis and sickle cell anemia. Dermatologic disease includes psoriasis, psoriatic arthritis and alopecia. Neurologic disease includes neurodegenerative diseases (multiple sclerosis, migraine, headache, amyloid-associated pathologies, prion diseases/Creutzfeld-Jacob disease, Alzheimer and Parkinson's diseases, multiple sclerosis, amyotrophic emilateral sclerosis) and peripheral neuropathies, migraine, headache. Renal disease includes nephrotic syndrome, hemodialysis and uremia. Iatrogenic intoxication condition includes OKT3 therapy, Anti-CD3 therapy, Cytokine therapy, Chemotherapy, Radiation therapy and chronic salicylate intoxication. Metabolic and idiopathic disease includes Wilson's disease, hemochromatosis, alpha-1 antitrypsin deficiency, diabetes, weight loss, anorexia, cachexia, obesity, Hashimoto's thyroiditis, osteoporosis, hypothalamic-pituitary-adrenal axis evaluation and primary biliary cirrhosis. Ophtalmological disease include glaucoma, retinopathies and dry-eye. A miscellanea of other pathologies comprehends: multiple organ dysfunction syndrome, muscular dystrophy, septic meningitis, atherosclerosis, epiglottitis, Whipple's disease, asthma, allergy, allergic rhinitis, organ necrosis, fever, septicaemia, endotoxic shock, hyperpyrexia, eosinophilic granuloma, granulomatosis, sarcoidosis, septic abortion, urethritis, emphysema, rhinitis, alveolitis, bronchiolitis, pharyngitis, epithelial barrier dysfunctions, pneumoultramicropicsilicovolcanoconiosis, pleurisy, sinusitis, influenza, respiratory syncytial virus infection, disseminated bacteremia, hydatid cyst, dermatomyositis, burns, sunburn, urticaria, warst, wheal, vasulitis, angiitis, myocarditis, arteritis, periarteritis nodosa, rheumatic fever, celiac disease, encephalitis, cerebral embolism, Guillame-Barre syndrome,

neuritis, neuralgia, iatrogenic complications/peripheral nerve lesions, spinal cord injury, paralysis, uveitis, arthriditis, arthralgias, osteomyelitis, fasciitis, Paget's disease, gout, periodontal disease, synovitis, myasthenia gravis, Goodpasture's syndrome, Babcets's syndrome, ankylosing spondylitis, Barger's disease, Retier's syndrome, bullous dermatitis (bullous pemphigoid), pemphigous and pemphigous vulgaris and alopecia.

In a further aspect of the invention, the use of the polypeptide variants obtained through systematic mutations of amino acid sequences of human and non-human Box-A of HMGB1, or of its biologically relevant fragments described above, is in combination with a further agent.

The further agent is preferably an agent capable of inhibiting an early mediator of the inflammatory cytokine cascade. Preferably, this further agent is an antagonist or inhibitor of a cytokine selected from the group consisting of TNF, IL-1α, IL-1β, IL-Ra, IL-6, IL-8, IL-10, IL 13, IL-18, IFN-γ MIP-1α, MIF-1β, MIP-2, MIF and PAF.

The further agent used in combination with the polypeptide variants of HMGB1 Box-A, or of its biologically relevant fragments, may also be an inhibitor of RAGE, e.g. an antibody directed to RAGE, a nucleic acid or nucleic acid analogue capable of inhibiting RAGE expression, e.g. an antisense molecule, a ribozyme or a RNA interference molecule, or a small synthetic molecule antagonist of the interaction of HMGB1 with RAGE, preferably of the interaction of the non-acetylated or/and acetylated form of HMGB1 with RAGE, or soluble RAGE (sRAGE). The antibody to RAGE is preferably a monoclonal antibody, more preferably a chimeric or humanised antibody or a recombinant antibody, such as a single chain antibody or an antigen-binding fragment of such an antibody. The soluble RAGE analog may be optionally present as a fusion protein, e.g. with the Fc domain of a human antibody. The small synthetic molecular antagonist of the HMGB1 interaction with RAGE preferably has a molecular weight of less than 1000

Dalton. The small synthetic molecular antagonist preferably inhibits the interaction of RAGE with the non-acetylated form or/and with the acetylated form of HMGB1 and with the non-acetylated form or/and with the acetylated form of HMGB1 homologous proteins, particularly HMGB2, HMGB3, HMG-1L10, HMG-4L or/and SP100-HMG.

The further agent used in combination with the polypeptide variants of HMGB1 Box-A, or of its biologically relevant fragments, may also be an inhibitor of the interaction of a Toll-like receptor (TLR), e.g. of TLR2, TLR4, TLR7, TLR8 or/and TLR9, with HMGB1, which inhibitor is preferably a monoclonal or polyclonal antibody, a nucleic acid or nucleic acid analogue capable of inhibiting TLR expression, e.g. an antisense molecule, a ribozyme or a RNA interference molecule, or a synthetic molecule preferably having a size of less than 1000 Dalton. The inhibitor may be a known inhibitor of a Toll-like receptor, in particular of TLR2, TLR4, TLR7, TLR8 or/and TLR9. The inhibitor preferably inhibits the interaction of the Toll-like receptor with the non-acetylated form or/and the acetylated form of HMGB1 and with the non-acetylated form or/and with the acetylated form of HMGB1 homologous proteins, in particular HMGB2, HMGB3, HMG-1L10, HMG-4L or/and SP100-HMG.

In still another embodiment, the further agent used in combination with the polypeptide variants of HMGB1 Box-A, or of its biologically relevant fragments, is the functional N-terminal lectin-like domain (D1) of thrombomodulin. The D1 domain of thrombomodulin is able to intercept the non-acetylated form and/or the acetylated form of released HMGB1 and of released HMGB1 homologous proteins, in particular HMGB2, HMGB3, HMG-1L10, HMG-4L or/and SP100-HMG, preventing thus their interaction with RAGE and Toll-like receptors. The D1 domain of thrombomodulin may be native or mutated in order to make it resistant to proteases.

The further agent may also be a synthetic double-stranded nucleic acid or

nucleic acid analogue molecule with a bent shape structure, particularly a double-stranded bent DNA, PNA or DNA/PNA chimera or hybrid or a double-stranded cruciform DNA, PNA or DNA/PNA chimera or hybrid structure, capable of binding to the HMGB1 protein. Preferred nucleic acids and nucleic analogue molecules are disclosed in a co-owned and co-pending international patent application No. PCT/EP2005/007198 filed on 4 July 2005 (claiming the priority of US provisional application No. 60/584,678 filed on 2 July 2004), which are incorporated herein by reference. The synthetic double-stranded nucleic acid or nucleic acid analogue molecule with a bent shape structure is preferably capable of binding to the non-acetylated or/and to the acetylated form of HMGB1 and the non-acetylated or/and the acetylated form of HMGB1 homologous proteins, in particular HMGB2, HMGB3, HMG-1L10, HMG4L or/and SP100-HMG.

In a still further embodiment, the further agent used in combination with the inventive polypeptide variants is K-252a or/and a salt or derivative thereof or a polymer conjugate of K-252a or/and of a derivative thereof. The use of K-252a or polymer conjugate of K-252a and derivatives thereof is disclosed in a co-owned and co-pending international patent application No. PCT/EP2005/008258 and US provisional application filed on 25 August 2005.

Therefore, a further aspect of the present invention is a pharmaceutical composition comprising an effective amount of at least one of the polypeptide variants of HMGB1 Box-A or a biologically active fragment thereof as an active ingredient for the treatment of HMGB1-associated pathologies and pharmaceutically acceptable carriers, diluents and/or adjuvants. The pharmaceutical composition of the present invention is preferably suitable for the treatment of pathologies associated with the non-acetylated or/and the acetylated form of HMGB1 and/or of HMGB1 homologous proteins. In a further preferred embodiment, the pharmaceutical composition of the present invention comprising the at least one polypeptide variant also comprises a further agent as defined above. The

pharmaceutical composition of the present invention may be used for diagnostic or for therapeutic applications.

The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's conditions. Administration may be achieved in a single dose or repeated doses at intervals. Dosage amount and interval may be adjusted individually in order to provide the therapeutical effect which results in amelioration of symptoms or a prolongation of the survival in a patient. The actual amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgement of the prescribing physician. A suitable daily dosage will be between 0,001 to 10 mg/kg, particularly 0,1 to 5 mg/kg.

The administration may be carried out by known methods, e.g. by injection, in particular by intravenous, intramuscular, transmucosal, subcutaneous or intraperitoneal injection and/or by oral, topical, nasal, inhalation, aerosol and/or rectal application, etc. The administration may be local or systemic.

In addition, the variants of Box-A of HMGB1, or of its pharmacologically active fragments, object of this invention can be reversibly immobilized and/or adsorbed on the surface and/or inside medical devices or drug release/vehicling systems (microspheres). Medical devices and microspheres can be reversibly loaded with the polypeptide variants of Box-A object of this invention, through their binding, impregnation and/or adsorption on the surface of the medical device or of the microsphere or on a layer that coats its surface. When the medical device or the microsphere come into contact with biological fluids, the reversibly immobilized variant of Box-A is released. Therefore, the medical device and the microsphere act as drug-releasing tools that elute the molecule object of this invention in such a way that their release kinetics can be controlled, ensuring controlled or

sustained release, as required by the treatment. The methods for coating/impregnating the medical devices and loading microspheres are well known by experts in these technologies.

Thus, a further aspect of this invention is the way of using the variants of Box-A of HMGB1 or its pharmacologically relevant fragments, wherein the mutated polypeptide molecules are reversibly immobilized on the surface of medical devices or of microspheres or are adsorbed within them. These medical instruments are preferably surgical tools, implants, catheters or stents, for example stents for angioplasty and, in particular, medicated drug-eluting stents.

Another aspect of the invention concerns a medical device reversibly coated with at least one polypeptide variant of the invention. Such a device can be selected from surgical instruments, implants, catheters or stents. Such a device may be useful for angioplasty.

The invention is further illustrated by the following Figures and Examples. The examples are intended to exemplify generic processes and are included for illustrative purpose only, without intention of limiting the scope of the present invention.

Fig. 1 shows the proteases used for the *in silico* identification of the amino acid positions (is-HITs) on the HMGB1 Box-A amino acid sequence which are targets for the proteolytic activity.

Fig. 2 depicts the "Percent Accepted Mutation" (PAM 250) matrix. Values given to identical residues are shown in grey square. Highest values in the matrix are shown in black square and correspond to the highest occurrence of substitution between two residues.

Fig. 3a displays the amino acid sequence of the native Human HMGB1 Box-A made of 84 amino acid residues. In bold, the amino acids sensitive to

proteases proteolysis are identified, showing the is-HIT residue positions.

Fig. 3b shows the type of replacing amino acids on the respective is-HITs target positions selected to generate the polypeptide variant of the full-length human HMGB1 Box-A. Further, the specific amino acid sequences of the generated polypeptide variant are displayed in SEQ ID NOs:2 to 116.

Fig. 4a displays the amino acid sequence of the biologically active fragment of Human HMGB1 Box-A made of 77 amino acid residues. In bold, the amino acids sensitive to proteases proteolysis are identified, showing the is-HIT residue positions.

Fig. 4b shows the type of replacing amino acids on the respective is-HITs target positions selected to generate the polypeptide variant of the biologically active fragment of Human HMGB1 Box-A made of 77 amino acid residues. Further the specific amino acid sequences of the generated polypeptide variant are displayed in SEQ ID NOs: 118 to 222.

Fig. 5a displays the amino acid sequence of the biologically active fragment of Human HMGB1 Box-A made of 54 amino acid residues. In bold, the amino acids sensitive to proteases proteolysis are identified, showing the is-HIT residue positions.

Fig. 5b shows the type of replacing amino acids on the respective is-HITs target positions selected to generate the polypeptide variant of the biologically active fragment of Human HMGB1 Box-A made of 54 amino acid residues. Further, the specific amino acid sequences of the generated polypeptide variant are displayed in SEQ ID NOs: 224 to 300.

Fig. 6a displays the amino acid sequence of the native *Anopheles gambia* HMGB1 Box-A made of 84 amino acid residues. In bold, the amino acids sensitive to proteases proteolysis are identified, showing the is-HIT residue positions.

Fig. 6b shows the type of replacing amino acids on the respective is-HITs target positions selected to generate the polypeptide variant of the full-length *Anopheles gambia* HMGB1 Box-A. Further, the specific amino acid sequences of the generated polypeptide variant are displayed in SEQ ID NOs: 302 to 419.

Fig. 7a displays the amino acid sequence of the biologically active fragment of *Anopheles gambia* HMGB1 Box-A made of 77 amino acid residues. In bold, the amino acids sensitive to proteases proteolysis are identified, showing the is-HIT residue positions.

Fig. 7b shows the type of replacing amino acids on the respective is-HITs target positions selected to generate the polypeptide variant of the biologically active fragment of *Anopheles gambia* HMGB1 Box-A made of 77 amino acid residues. Further the specific amino acid sequences of the generated polypeptide variant are displayed in SEQ ID NOs: 420 to 529.

Fig. 8a displays the amino acid sequence of the biologically active fragment of *Anopheles gambia* HMGB1 Box-A made of 54 amino acid residues. In bold, the amino acids sensitive to proteases proteolysis are identified, showing the is-HIT residue positions.

Fig. 8b shows the type of replacing amino acids on the respective is-HITs target positions selected to generate the polypeptide variant of the biologically active fragment of *Anopheles gambia* HMGB1 Box-A made of 54 amino acid residues. Further, the specific amino acid sequences of the generated polypeptide variant are displayed in SEQ ID NOs: 531 to 612.

Fig. 9 shows the plasmid vector containing the nucleic acid sequence encoding for the polypeptide variant of the present invention. The plasmid contains the gene encoding for the polypeptide variant of the present invention, which is under control of the IPTG inducible T5 promoter. The

plasmid further contains an ampicillin resistant gene, a 6x His-tag and several restriction sites.

Fig. 10 shows a graph displaying the correlation between the TNF-alpha release induced by the stimulation of HMGB1 in RAW 264.7 cells.

Fig. 11 displays a dose-dependent inhibition of HMGB1-induced TNF-alpha release by a Box-A His-tagged protein.

EXAMPLES

1. PRODUCTION OF HMGB1 BOX-A NATIVE AND VARIANTS IN BACTERIA

The *in silico* generated variants of HMGB1 Box-A were cloned from HMGB1 protein into an inducible plasmid vector (Fig. 9) used to transform E. coli M15 strain competent cells. M15 cells were grown overnight in 1 mL of LB medium containing Kanamicyn and Ampicillin in 96 deep-well plates under agitation (750 rpm). At $OD_{600\,nm}$ of 0.2-0.3 the cultures were diluted in 5 mL of LB medium in 24-well plates to reach an $OD_{600\,nm}$ of 0.07.

The M15 cells were incubated at 37°C under constant agitation (200 rpm). The production of Box-A (native or variants) was induced by the addition of IPTG (1mM final concentration) at $OD_{600\,nm}$ of 0.6. The culture was continued for three hours at 37°C under agitation (200 rpm). M15 cells were then harvested by centrifugation at 1000 g for 15 minutes, the supernatant was discarded and the pellet stored at –80°C at least for 1 hour before cells lysis and Box-A purification.

2. PURIFICATION OF HMGB1 BOX A NATIVE AND VARIANTS

M15 cells pellet was thawed on ice for 15 min. The cells were resuspended in 1 mL NPI-10 buffer containing 1 mg/mL Lysozyme and incubated for 30 min at RT under agitation at 750 rpm on a plate shaker. After the equilibration of Ni-NTA QIAfilter with 200 µL of Superflow resin (QIAGEN catalog#969261) and 600 µL of NPI-10 buffer the bacterial lysate was loaded and 200 µL of absolute EtOH added. Four wash steps with 1 mL of NPI-20 were performed. The second and third washes were done with 1mL NPI-20 added with 100 µg/mL Polymyxin (Fluka catalog#81271) in order to deplete LPS contaminants. After wash steps Box-A native and variants were eluted with 450 µL NPI-250. The samples were stored at 4°C.

Box-A native and variants were re-purified with a DetoxiGel polymyxin column (PIERCE) at 4°C according to the supplier instructions. Finally the eluted proteins were filtered (0.22 µm) in PBS and stored at 4°C to be tested.

3. BOX-A BIOLOGICAL ACTIVITY ASSAY

HMGB1 stimulates the secretion of TNF-alpha and of other cytokines as well as the proliferation of macrophages and monocytes. Box-A acts as an antagonist by inhibiting the activity of HMGB1.

The activity of Box-A native and variants produced were measured by the level of inhibition on the stimulation produced by HMGB1 on RAW 264.7 cells (murine macrophages, ATCC).

HMGB1 Box-A native and variants produced as described above were tested in a two-step process of screening directed to test i) their inhibition of HMGB1 induced TNF-alpha release and ii) their resistance to proteolysis.

In order to determine the proper HMGB1 concentration to be used in inhibition assay RAW 264.7 cells were seeded in 96 well plates ($4x10^5$ cells/well) and grown overnight in RPMI 1640 medium supplemented with

0.1% BSA. After overnight culture, cells were stimulated with HMGB1 (two times serial dilution concentrations between 100 µg/mL and 0.05 µg/mL) for 24 hours. The level of TNF-alpha produced was measured from cell media using ELISA (R&D systems), according to the manufacturer instructions. As presented in Fig. 10, HMGB1 significantly stimulated TNF-alpha release in macrophage cultures.

4. BOX-A INHIBITION OF HMGB1 TNF-ALPHA RELEASE AS SCREENING TEST

Murine macrophage-like RAW 264.7 cells were seeded in 96 well plates ($4x10^5$ cells/well) and grown overnight in RPMI 1640 medium supplemented with 0.1% BSA. After overnight culture, cells were stimulated with an adequate concentration of HMGB1 and Box-A native or variants or His-tagged (two times serial dilution between 20 µg/mL and 0.5 µg/mL) for 24 hours. The level of TNF-alpha was measured from cell media using ELISA (R&D systems), according to the manufacturer instructions.

Fig. 11 shows an example of dose-dependent inhibition of HMGB1 induced TNF release by Box-A, with an EC50 of 7.5 µg/ml (solid line). 100% inhibition of TNF-alpha release is obtained with a concentration of 20 µg/ml of Box-A. In parallel, TNF-alpha levels are measured in Box-A stimulated cells without HMGB1 in order to determine the presence or absence of contaminating endotoxin in Box-A preparation and quantify any non-HMGB1 dependent release of TNF-alpha. No release of TNF-alpha is observed at all concentrations of Box-A used in the assay (dashed line).

5. RESISTANCE TO PROTEOLYSIS OF BOX-A VARIANTS

Resistance of Box-A variants to proteolysis is determined as the residual biological activity (in the HMGB1/RAW cells system) following exposure to a mixture of selected proteases at increasing times of incubation.

20 µg of Box-A native or variants were treated with a mixture of proteases at 1% w/w of total proteins. The mixture of proteases was freshly prepared for each assay from stock solutions of endoproteinase Glu-C (SIGMA; 200 µg/ml), trypsin (SIGMA; 400µg/ml) and α-chymotrypsin (SIGMA; 400 µg/ml).

Samples were collected at different time points between 5 minutes and 8 hours of incubation with proteases after stopping the reaction with the addition of 10 µl of anti-proteases solution (Roche). Biological activity of each sample was then evaluated by the screening test described above in order to assess the residual activity at each time point.

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Claims

1. Polypeptide variant of the human and/or non human HMGB1 high affinity binding domain Box-A (HMGB1 Box-A) or of a biologically active fragment of HMGB1 Box-A, characterised in that the amino acid sequence of said polypeptide variant differs from the amino acid sequence of the wild type HMGB1 Box-A by the mutation of one or more single amino acid.

2. Polypeptide variant of claim 1, wherein the polypeptide variant differs from the wild type HMGB1 Box-A sequence by the mutation of 1 to 10 single amino acid, preferably by only one single amino acid.

3. Polypeptide variant of claim 1 or claim 2, wherein the mutation is a substitution, a deletion or an addition of single amino acids.

4. Polypeptide variant of claim 3, wherein the substitution is obtained by different genetically encoded amino acid or by non-genetically encoded amino acids.

5. Polypeptide variant of claim 3 or 4, wherein the substitution is a conservative or a non-conservative substitution.

6. Polypeptide variant of any of the preceding claims, wherein non-human HMGB1 Box-A is *Anopheles gambia* HMGB1 Box-A.

7. Polypeptide variant of any of the preceding claims, wherein the polypeptide variant of the human HMGB1 Box-A is selected from the group consisting of the amino acid sequences as defined in any of SEQ ID NO:2 to 116.

8. Polypeptide variant of any of the preceding claims, wherein the

biologically active fragments of the human wild type HMGB1 Box-A is a fragment of at least 77 or at least 54 amino acids respectively and comprises the amino acid sequences as defined in SEQ ID NO:117 or 223 respectively.

9. Polypeptide variant of claim 7 or 8, wherein the polypeptide variant of the biologically active fragments of the human HMGB1 Box-A is selected from the group consisting of the amino acid sequences as defined in any of SEQ ID NO:118 to 222 or 224 to 300.

10. Polypeptide variant of any of claims 1 to 6, wherein the polypeptide variant of the *Anopheles gambia* HMGB1 Box-A is selected from the group consisting of the amino acid sequences as defined in any of SEQ ID NO:302 to 418.

11. Polypeptide variant of any of claims 1 to 6, wherein the biologically active fragments of the *Anopheles gambia* wild type HMGB1 Box-A is a fragment of at least 77 or at least 54 amino acids respectively and comprises the amino acid sequences as defined in SEQ ID NO:419 or 530 respectively.

12. Polypeptide variant of claim 11, wherein the polypeptide variant of the biologically active fragments of the *Anopheles gambia* HMGB1 Box-A is selected from the group consisting of the amino acid sequences as defined in any of SEQ ID NO:420 to 529 or 531 to 612.

13. Polypeptide variant of any of claims 1 to 12, wherein said polypeptide variant exhibits an increased resistance to the proteolytic activity of proteases compared to the wild type HMGB1 Box-A or to the biologically active fragment of the wild type HMGB1 Box-A.

14. Polypeptide variant of any of the preceding claims, wherein the increase in resistance to proteolysis is in respect to at least one protease

selected from the group comprising chymotrypsin, trypsin, endoprotease, endopeptidase or a combination thereof.

15. Polypeptide variant of any of the preceding claims, wherein the increase in resistance to proteolysis is at least 10%, 20%, 30%, 40%, 50%, 70%, 80%, 90%, 95% or more compared to the wild type HMGB1 Box-A.

16. Polypeptide variant of any of the preceding claims, wherein the polypeptide variant exhibits a longer half life in body fluids compared to the wild type HMGB1 Box-A or to the biologically active fragment of the wild type HMGB1 Box-A.

17. Polypeptide variant of claim 16, wherein the half life is at least 10 minutes, 20 minutes, 30 minutes, 60 minutes or even longer compared to the wild type HMGB1 Box-A.

18. A nucleic acid molecule encoding a polypeptide variant as defined in any of claims 1 to 17.

19. A vector comprising a nucleic acid molecule of claim 18.

20. A method for producing a polypeptide variant of any of claims 1 to 17, comprising:
(i) introducing a nucleic acid molecule of claim 18 into a host; and
(ii) culturing the cell, under conditions in which the encoded polypeptide variant is expressed.

21. A method for producing a polypeptide variant of claims 1 to 17 using chemical peptide synthesis.

22. Polypeptide variant of any of claims 1 to 17 for the use as active agent in a medicament.

23. Use of a polypeptide variant of any of claims 1 to 17 for the manufacture of a medicament for the prevention or treatment of HMGB1-associated pathologies or pathologies associated with HMGB1 homologous proteins.

24. The use of claim 23, wherein the HMGB1-associated pathologies and the pathologies associated with HMGB1 homologous proteins are pathological conditions mediated by activation of the inflammatory cytokine cascade.

25. The use of claim 23 or 24, wherein the pathological conditions are selected from the group consisting of inflammatory disease, autoimmune disease, systemic inflammatory response syndrome, reperfusion injury after organ transplantation, cardiovascular affections, obstetric and gynecologic disease, infectious (viral and bacterial) disease, allergic and atopic disease, solid and liquid tumor pathologies, transplant rejection diseases, congenital diseases, dermatological diseases, neurological diseases, cachexia, renal diseases, iatrogenic intoxication conditions, metabolic and iodiopathic diseases, and ophthalmological diseases.

26. The use of any one of claims 23 to 25 in combination with a further agent capable of inhibiting an early mediator of the inflammatory cytokine cascade.

27. The use of claim 26, wherein the further agent is an antagonist or inhibitor of a cytokine selected from the group consisting of TNF, IL-1α, IL-1β, IL-R_a, IL-6, IL-8, IL-10, IL-13, IL-18, IFN-γ, MIP-1α, MIF-1β, MIP-2, MIF and PAF.

28. The use of any of claims 26, wherein the further agent is an antibody to RAGE, a nucleic acid or nucleic acid analogue capable of inhibiting RAGE expression, e.g. an antisense molecule, a ribozyme or a RNA

interference molecule, or a small synthetic molecule antagonist of the HMGB1 interaction with RAGE or soluble RAGE (sRAGE).

29. The use of any of claims 26, wherein the further agent which is an inhibitor of the interaction of a Toll-like receptor (TLR), in particular of TLR2, TLR4, TLR7, TLR8 or/and TLR9, with HMGB1, preferably a monoclonal or polyclonal antibody, a nucleic acid or nucleic acid analogue capable of inhibiting TLR expression, e.g. an antisense molecule, a ribozyme or a RNA interference molecule, or a synthetic molecule having a size of less than 1000 Dalton.

30. The use of any of claims 26 wherein the further agent is the N-terminal lectin-like domain (D1) of native or mutated thrombomodulin.

31. The use of claim 26, wherein the further agent is a synthetic double-stranded nucleic acid or nucleic acid analogue molecule with a bent shape structure, selected from bent or cruciform DNA, PNA or DNA/PNA chimeria or hybrid.

32. The use of claim 26, wherein the further agent is K-252a or/and a salt or a derivative thereof or a polymer conjugate of K-252a or/and a derivative thereof.

33. A pharmaceutical composition comprising an effective amount of at least one polypeptide variant of any of claims 1 to 17 as an active agent and optionally a pharmaceutically acceptable carrier.

34. The composition of claims 33 wherein the at least one polypeptide variant is in combination with at least one further agent as defined in any one of claims 27 to 32.

35. The composition of claims 33 or 34 for diagnostic applications.

36. The composition of claims 33 to 34 for therapeutic applications.

37. A method of treating a condition in a patient, characterized by HMGB1-activation of an inflammatory cytokine cascade, comprising administering to the patient an effective amount of at least one of the polypeptide variants of any one of claims 1 to 17, capable of antagonize and/or inhibit the pathological activity induced by HMGB1.

38. The use of at least one polypeptide variant of any one of claims 1 to 17, wherein said molecules are reversibly immobilised on the surface of medical devices.

39. The use of claim 38, wherein said medical devices are surgical instruments, implants, catheters or stents.

40. Medical device reversibly coated with at least one polypeptide variant of any one of claims 1 to 17.

41. Medical device of claim 40, wherein the medical device is selected from surgical instruments, implants, catheters or stents.

Figure 1

In silico identification of all amino acid positions that are targets for proteolysis using a large number of selected proteases and chemical treatments.

AspN	'D	Endoproteinase Asp-N
Chymo	(F,W,Y,M,L)'~P	Chymotrypsin
Clos	R'	Clostripain
CnBr	M'	Cyanogen Bromide
IbzO	W'	IodosoBenzoate
Myxo	K'	Myxobacter
NH2OH	N'G	Hydroxylamine
pH2.5	D'P	pH 2.5
ProEn	P'	Proline Endopeptidase
Staph	E'	Staphylococcal Protease
Tryp	(K,R)'~P	Trypsin
TrypK	K'~P	Trypsin(Arg blocked)
TrypR	R'~P	Trypsin(Lys blocked)

Figure 2 – Percent Accepted Mutation (PAM 250)

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	2	-2	0	0	-2	0	0	1	-1	-1	-2	-1	-1	-3	1	1	1	-6	-3	0
R	-2	6	0	-1	-4	1	-1	-3	2	-2	-3	3	0	-4	0	0	-1	2	-4	-2
N	0	0	2	2	-4	1	1	0	2	-2	-3	1	-2	-3	0	1	0	-4	-2	-2
D	0	-1	2	4	-5	2	3	1	1	-2	-4	0	-3	-6	-1	0	0	-7	-4	-2
C	-2	-4	-4	-5	12	-5	-5	-3	-3	-2	-6	-5	-5	-4	-3	0	-2	-8	0	-2
Q	0	1	1	2	-5	4	2	-1	3	-2	-2	1	-1	-5	0	-1	-1	-5	-4	-2
E	0	-1	1	3	-5	2	4	0	1	-2	-3	0	-2	-5	-1	0	0	-7	-4	-2
G	1	-3	0	1	-3	-1	0	5	-2	-3	-4	-2	-3	-5	0	1	0	-7	-5	-1
H	-1	2	2	1	-3	3	1	-2	6	-2	-2	0	-2	-2	0	-1	-1	-3	0	-2
I	-1	-2	-2	-2	-2	-2	-2	-3	-2	5	2	-2	2	1	-2	-1	0	-5	-1	4
L	-2	-3	-3	-4	-6	-2	-3	-4	-2	2	6	-3	4	2	-3	-3	-2	-2	-1	2
K	-1	3	1	0	-5	1	0	-2	0	-2	-3	5	0	-5	-1	0	0	-3	-4	-2
M	-1	0	-2	-3	-5	-1	-2	-3	-2	2	4	0	6	0	-2	-2	-1	-4	-2	2
F	-3	-4	-3	-6	-4	-5	-5	-5	-2	1	2	-5	0	9	-5	-3	-3	0	7	-1
P	1	0	0	-1	-3	0	-1	0	0	-2	-3	-1	-2	-5	6	1	0	-6	-5	-1
S	1	0	1	0	0	-1	0	1	-1	-1	-3	0	-2	-3	1	2	1	-2	-3	-1
T	1	-1	0	0	-2	-1	0	0	-1	0	-2	0	-1	-3	0	1	3	-5	-3	0
W	-6	2	-4	-7	-8	-5	-7	-7	-3	-5	-2	-3	-4	0	-6	-2	-5	17	0	-6
Y	-3	-4	-2	-4	0	-4	-4	-5	0	-1	-1	-4	-2	7	-5	-3	-3	0	10	-2
V	0	-2	-2	-2	-2	-2	-2	-1	-2	4	2	-2	2	-1	-1	-1	0	-6	-2	4

Value given for identical residues.

Positive value of substitution between two residues.

Figure 3a

Box A 84 amino acids

Protection against proteolysis
If sequence:

GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKE
KGKFEDMAKADKARYEREMKTYIPPKGET

In bold amino acids sensitive to proteases proteolysis

Figure 3b

Box A 84 amino acids

 # Mutant list:

K2N	K27Q	E55Q	E73N
K2Q	K28N	E55H	M74I
D4N	K28Q	E55N	M74V
D4Q	K29N	K56N	K75N
P5A	K29Q	K56Q	K75Q
P5S	P31A	K58N	Y77H
K6N	P31S	K58Q	Y77I
K6Q	D32N	F59I	P79A
K7N	D32Q	F59V	P79S
K7Q	F37I	E60Q	P80A
P8A	F37V	E60H	P80S
P8S	E39Q	E60N	K81N
R9H	E39H	D61N	K81Q
R9Q	E39N	D61Q	E83Q
K11N	F40I	M62I	E83H
K11Q	F40V	M62V	E83N
M12I	K42N	K64N	
M12V	K42Q	K64Q	
Y15H	K43N	D66N	
Y15I	K43Q	D66Q	
F17I	E46Q	K67N	
F17V	E46H	K67Q	
F18I	E46N	R69H	
F18V	R47H	R69Q	
R23H	R47Q	Y70H	
R23Q	W48Y	Y70I	
E24Q	W48S	E71Q	
E24H	K49N	E71H	
E24N	K49Q	E71N	
E25Q	M51I	R72H	
E25H	M51V	R72Q	
E25N	K54N	E73Q	
K27N	K54Q	E73H	

Figure 3b continued

Box A 84 amino acid sequences:

> sequence 1 Wild type
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 2 K2N
GNGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 3 K2Q
GQGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 4 D4N
GKGNPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 5 D4Q
GKGQPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 6 P5A
GKGDAKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 7 P5S
GKGDSKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 8 K6N
GKGDPNKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 9 K6Q
GKGDPQKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 10 K7N
GKGDPKNPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 11 K7Q
GKGDPKQPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 12 P8A
GKGDPKKARGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 13 P8S
GKGDPKKSRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKE

Figure 3b continued

KGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 14 R9H
GKGDPKKPHGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 15 R9Q
GKGDPKKPQGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 16 K11N
GKGDPKKPRGNMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 17 K11Q
GKGDPKKPRGQMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 18 M12I
GKGDPKKPRGKISSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADK
ARYEREMKTYIPPKGET

> sequence 19 M12V
GKGDPKKPRGKVSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADK
ARYEREMKTYIPPKGET

> sequence 20 Y15H
GKGDPKKPRGKMSSHAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 21 Y15I
GKGDPKKPRGKMSSIAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADK
ARYEREMKTYIPPKGET

> sequence 22 F17I
GKGDPKKPRGKMSSYAIFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADK
ARYEREMKTYIPPKGET

> sequence 23 F17V
GKGDPKKPRGKMSSYAVFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 24 F18I
GKGDPKKPRGKMSSYAFIVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADK
ARYEREMKTYIPPKGET

> sequence 25 F18V
GKGDPKKPRGKMSSYAFVVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 26 R23H
GKGDPKKPRGKMSSYAFFVQTCHEEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

Figure 3b continued

> sequence 27 R23Q
GKGDPKKPRGKMSSYAFFVQTCQEEHKKKHPDASVNFSEFSKKCSERWKTMSAKEGKFEDMAKADK

AYEREMKTYIPPKKGET

> sequence 28 E24Q
GKGDPKKPRGKMSSYAFFVQTCRQEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 29 E24H
GKGDPKKPRGKMSSYAFFVQTCRHEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 30 E24N
GKGDPKKPRGKMSSYAFFVQTCRNEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 31 E25Q
GKGDPKKPRGKMSSYAFFVQTCREQHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 32 E25H
GKGDPKKPRGKMSSYAFFVQTCREHHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 33 E25N
GKGDPKKPRGKMSSYAFFVQTCRENHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 34 K27N
GKGDPKKPRGKMSSYAFFVQTCREEHNKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 35 K27Q
GKGDPKKPRGKMSSYAFFVQTCREEHQKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 36 K28N
GKGDPKKPRGKMSSYAFFVQTCREEHKNKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 37 K28Q
GKGDPKKPRGKMSSYAFFVQTCREEHKQKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 38 K29N
GKGDPKKPRGKMSSYAFFVQTCREEHKKNHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 39 K29Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKQHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

Figure 3b continued

> sequence 40 P31A
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHADASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 41 P31S
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHSDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 42 D32N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPNASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 43 D32Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPQASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 44 F37I
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNISEFSKKCSERWKTMSAKEKGKFEDMAKADK
ARYEREMKTYIPPKGET

> sequence 45 F37V
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNVSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 46 E39Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSQFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 47 E39H
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSHFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 48 E39N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSNFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 49 F40I
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEISKKCSERWKTMSAKEKGKFEDMAKADK
ARYEREMKTYIPPKGET

> sequence 50 F40V
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEVSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 51 K42N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSNKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 52 K42Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSQKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 53 K43N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKNCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

Figure 3b continued

> sequence 54 K43Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKQCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 55 E46Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSQRWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 56 E46H
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSHRWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 57 E46N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSNRWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 58 R47H
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSEHWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 59 R47Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSEQWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 60 W48Y
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERYKTMSAKEKGKFEDMAKADK
ARYEREMKTYIPPKGET

> sequence 61 W48S
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERSKTMSAKEKGKFEDMAKADK
ARYEREMKTYIPPKGET

> sequence 62 K49N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWNTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 63 K49Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWQTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 64 M51I
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTISAKEKGKFEDMAKADK
ARYEREMKTYIPPKGET

> sequence 65 M51V
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTVSAKEKGKFEDMAKADK
ARYEREMKTYIPPKGET

> sequence 66 K54N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSANEKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 67 K54Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAQEKGKFEDMAKAD

Figure 3b continued

KARYEREMKTYIPPKGET

> sequence 68 E55Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKQKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 69 E55H
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKHKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 70 E55N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKNKGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 71 K56N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKENGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 72 K56Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEQGKFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 73 K58N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGNFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 74 K58Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGQFEDMAKAD
KARYEREMKTYIPPKGET

> sequence 75 F59I
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKIEDMAKADK
ARYEREMKTYIPPKGET

> sequence 76 F59V
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKVEDMAKAD
KARYEREMKTYIPPKGET

> sequence 77 E60Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFQDMAKAD
KARYEREMKTYIPPKGET

> sequence 78 E60H
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFHDMAKAD
KARYEREMKTYIPPKGET

> sequence 79 E60N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFNDMAKAD
KARYEREMKTYIPPKGET

> sequence 80 D61N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFENMAKAD
KARYEREMKTYIPPKGET

> sequence 81 D61Q

Figure 3b continued 10/56

GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEQMAKAD
KARYEREMKTYIPPKGET

> sequence 82 M62I
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDIAKADK
ARYEREMKTYIPPKGET

> sequence 83 M62V
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDVAKADK
ARYEREMKTYIPPKGET

> sequence 84 K64N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMANAD
KARYEREMKTYIPPKGET

> sequence 85 K64Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAQAD
KARYEREMKTYIPPKGET

> sequence 86 D66N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAN
KARYEREMKTYIPPKGET

> sequence 87 D66Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAQ
KARYEREMKTYIPPKGET

> sequence 88 K67N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
NARYEREMKTYIPPKGET

> sequence 89 K67Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
QARYEREMKTYIPPKGET

> sequence 90 R69H
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KAHYEREMKTYIPPKGET

> sequence 91 R69Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KAQYEREMKTYIPPKGET

> sequence 92 Y70H
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARHEREMKTYIPPKGET

> sequence 93 Y70I
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARIEREMKTYIPPKGET

> sequence 94 E71Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYQREMKTYIPPKGET

Figure 3b continued

> sequence 95 E71H
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYHREMKTYIPPKGET

> sequence 96 E71N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYNREMKTYIPPKGET

> sequence 97 R72H
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEHEMKTYIPPKGET

> sequence 98 R72Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEQEMKTYIPPKGET

> sequence 99 E73Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYERQMKTYIPPKGET

> sequence 100 E73H
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYERHMKTYIPPKGET

> sequence 101 E73N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYERNMKTYIPPKGET

> sequence 102 M74I
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREIKTYIPPKGET

> sequence 103 M74V
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREVKTYIPPKGET

> sequence 104 K75N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMNTYIPPKGET

> sequence 105 K75Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMQTYIPPKGET

> sequence 106 Y77H
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTHIPPKGET

> sequence 107 Y77I
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTIIPPKGET

> sequence 108 P79A
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIAPKGET

Figure 3b continued

> sequence 109 P79S
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYISPKGET

> sequence 110 P80A
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPAKGET

> sequence 111 P80S
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPSKGET

> sequence 112K81N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPNGET

> sequence 113 K81Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPQGET

> sequence 114 E83Q
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGQT

> sequence 115 E83H
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGHT

> sequence 116 E83N
GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAD
KARYEREMKTYIPPKGNT

Figure 4a

Box A 77 amino acids

 # Protection against proteolysis
If sequence:

PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDM
AKADKARYEREMKTYIPPKGET

In bold amino acids sensitive to proteases proteolysis

Figure 4b

Box A 77 amino acids

 # Mutant list:

P1A	F30V	E53H	P73S
P1S	E32Q	E53N	K74N
R2H	E32H	D54N	K74Q
R2Q	E32N	D54Q	E76Q
K4N	F33I	M55I	E76H
K4Q	F33V	M55V	E76N
M5I	K35N	K57N	
M5V	K35Q	K57Q	
Y8H	K36N	D59N	
Y8I	K36Q	D59Q	
F10I	E39Q	K60N	
F10V	E39H	K60Q	
F11I	E39N	R62H	
F11V	R40H	R62Q	
R16H	R40Q	Y63H	
R16Q	W41Y	Y63I	
E17Q	W41S	E64Q	
E17H	K42N	E64H	
E17N	K42Q	E64N	
E18Q	M44I	R65H	
E18H	M44V	R65Q	
E18N	K47N	E66Q	
K20N	K47Q	E66H	
K20Q	E48Q	E66N	
K21N	E48H	M67I	
K21Q	E48N	M67V	
K22N	K49N	K68N	
K22Q	K49Q	K68Q	
P24A	K51N	Y70H	
P24S	K51Q	Y70I	
D25N	F52I	P72A	
D25Q	F52V	P72S	
F30I	E53Q	P73A	

Figure 4b continued

Box A 77 amino acid sequences

> sequence 117 Wild type

PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 118 P1A
ARGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 119 P1S
SRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 120 R2H
PHGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 121 R2Q
PQGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 122 K4N
PRGNMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 123 K4Q
PRGQMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 124 M5I
PRGKISSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 125 M5V
PRGKVSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 126 Y8H
PRGKMSSHAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 127 Y8I
PRGKMSSIAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 128 F10I
PRGKMSSYAIFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 129 F10V
PRGKMSSYAVFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE

Figure 4b continued

MKTYIPPKGET

> sequence 130 F11I
PRGKMSSYAFIVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 131 F11V
PRGKMSSYAFVVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 132 R16H
PRGKMSSYAFFVQTCHEEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 133 R16Q
PRGKMSSYAFFVQTCQEEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 134 E17Q
PRGKMSSYAFFVQTCRQEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 135 E17H
PRGKMSSYAFFVQTCRHEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 136 E17N
PRGKMSSYAFFVQTCRNEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 137 E18Q
PRGKMSSYAFFVQTCREQHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 138 E18H
PRGKMSSYAFFVQTCREHHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 139 E18N
PRGKMSSYAFFVQTCRENHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 140 K20N
PRGKMSSYAFFVQTCREEHNKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 141 K20Q
PRGKMSSYAFFVQTCREEHQKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 142 K21N
PRGKMSSYAFFVQTCREEHKNKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 143 K21Q
PRGKMSSYAFFVQTCREEHKQKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE

Figure 4b continued

MKTYIPPKGET

> sequence 144 K22N
PRGKMSSYAFFVQTCREEHKKNHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 145 K22Q
PRGKMSSYAFFVQTCREEHKKQHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 146 P24A
PRGKMSSYAFFVQTCREEHKKKHADASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 147 P24S
PRGKMSSYAFFVQTCREEHKKKHSDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 148 D25N
PRGKMSSYAFFVQTCREEHKKKHPNASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 149 D25Q
PRGKMSSYAFFVQTCREEHKKKHPQASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 150 F30I
PRGKMSSYAFFVQTCREEHKKKHPDASVNISEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 151 F30V
PRGKMSSYAFFVQTCREEHKKKHPDASVNVSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 152 E32Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSQFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 153 E32H
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSHFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 154 E32N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSNFSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 155 F33I
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEISKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 156 F33V
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEVSKKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 157 K35N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSNKCSERWKTMSAKEKGKFEDMAKADKARYEREM

Figure 4b continued

KTYIPPKGET

> sequence 158 K35Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSQKCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 159 K36N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKNCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 160 K36Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKQCSERWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 161 E39Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSQRWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 162 E39H
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSHRWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 163 E39N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSNRWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 164 R40H
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSEHWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 165 R40Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSEQWKTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 166 W41Y
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERYKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 167 W41S
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERSKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 168 K42N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWNTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 169 K42Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWQTMSAKEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 170 M44I
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTISAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 171 M44V

Figure 4b continued

PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTVSAKEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 172 K47N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSANEKGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 173 K47Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAQEKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 174 E48Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKQKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 175 E48H
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKHKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 176 E48N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKNKGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 177 K49N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKENGKFEDMAKADKARYEREM
KTYIPPKGET

> sequence 178 K49Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEQGKFEDMAKADKARYERE
MKTYIPPKGET

> sequence 179 K51N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGNFEDMAKADKARYEREM
KTYIPPKGET

> sequence 180 K51Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGQFEDMAKADKARYERE
MKTYIPPKGET

> sequence 181 F52I
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKIEDMAKADKARYEREM
KTYIPPKGET

> sequence 182 F52V
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKVEDMAKADKARYERE
MKTYIPPKGET

> sequence 183 E53Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFQDMAKADKARYERE
MKTYIPPKGET

> sequence 184 E53H
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFHDMAKADKARYERE
MKTYIPPKGET

Figure 4b continued

> sequence 185 E53N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFNDMAKADKARYERE
MKTYIPPKGET

> sequence 186 D54N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFENMAKADKARYEREM
KTYIPPKGET

> sequence 187 D54Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEQMAKADKARYERE
MKTYIPPKGET

> sequence 188 M55I
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDIAKADKARYEREM
KTYIPPKGET

> sequence 189 M55V
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDVAKADKARYEREM
KTYIPPKGET

> sequence 190 K57N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMANADKARYEREM
KTYIPPKGET

> sequence 191 K57Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAQADKARYERE
MKTYIPPKGET

> sequence 192 D59N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKANKARYEREM
KTYIPPKGET

> sequence 193 D59Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAQKARYERE
MKTYIPPKGET

> sequence 194 K60N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADNARYEREM
KTYIPPKGET

> sequence 195 K60Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADQARYERE
MKTYIPPKGET

> sequence 196 R62H
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKAHYEREM
KTYIPPKGET

> sequence 197 R62Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKAQYERE
MKTYIPPKGET

> sequence 198 Y63H
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARHERE
MKTYIPPKGET

Figure 4b continued

> sequence 199 Y63I
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARIEREM
KTYIPPKGET

> sequence 200 E64Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYQRE
MKTYIPPKGET

> sequence 201 E64H
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYHRE
MKTYIPPKGET

> sequence 202 E64N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYNRE
MKTYIPPKGET

> sequence 203 R65H
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEHEM
KTYIPPKGET

> sequence 204 R65Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEQE
MKTYIPPKGET

> sequence 205 E66Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERQ
MKTYIPPKGET

> sequence 206 E66H
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERH
MKTYIPPKGET

> sequence 207 E66N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERN
MKTYIPPKGET

> sequence 208 M67I
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREI
KTYIPPKGET

> sequence 209 M67V
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREV
KTYIPPKGET

> sequence 210 K68N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
NTYIPPKGET

> sequence 211 K68Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
QTYIPPKGET

> sequence 212 Y70H
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM

Figure 4b continued

KTHIPPKGET

> sequence 213 Y70I
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTIIPPKGET

> sequence 214 P72A
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIAPKGET

> sequence 215 P72S
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYISPKGET

> sequence 216 P73A
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPAKGET

> sequence 217 P73S
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPSKGET

> sequence 218 K74N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPNGET

> sequence 219 K74Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPQGET

> sequence 220 E76Q
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGQT

> sequence 221 E76H
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGHT

> sequence 222 E76N
PRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREM
KTYIPPKGNT

Figure 5a

Box A 54 amino acids

 # Protection against proteolysis
If sequence:

PDASVN**F**SE**F**S**KK**CSE**R**W**K**TMSA**K**E**K**G**K**F**ED**MA**K**AD**K**AR**Y**E**R**EM**K**TYIPP**K**GET

In bold amino acids sensitive to proteases proteolysis

Figure 5b

Box A 54 amino acids

 # Mutant list:

P1A	F29I	P50A
P1S	F29V	P50S
D2N	E30Q	K51N
D2Q	E30H	K51Q
F7I	E30N	E53Q
F7V	D31N	E53H
E9Q	D31Q	E53N
E9H	M32I	
E9N	M32V	
F10I	K34N	
F10V	K34Q	
K12N	D36N	
K12Q	D36Q	
K13N	K37N	
K13Q	K37Q	
E16Q	R39H	
E16H	R39Q	
E16N	Y40H	
R17H	Y40I	
R17Q	E41Q	
W18Y	E41H	
W18S	E41N	
K19N	R42H	
K19Q	R42Q	
M21I	E43Q	
M21V	E43H	
K24N	E43N	
K24Q	M44I	
E25Q	M44V	
E25H	K45N	
E25N	K45Q	
K26N	Y47H	
K26Q	Y47I	
K28N	P49A	
K28Q	P49S	

Figure 5b continued

Box A 54 amino acid sequences:

> sequence 223 Wild type

PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 224 P1A
ADASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 225 P1S
SDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 226 D2N
PNASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 227 D2Q
PQASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 228 F7I
PDASVNISEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 229 F7V
PDASVNVSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 230 E9Q
PDASVNFSQFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 231 E9H
PDASVNFSHFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 232 E9N
PDASVNFSNFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 233 F10I
PDASVNFSEISKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 234 F10V
PDASVNFSEVSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 235 K12N
PDASVNFSEFSNKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 236 K12Q
PDASVNFSEFSQKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 237 K13N
PDASVNFSEFSKNCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 238 K13Q
PDASVNFSEFSKQCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 239 E16Q
PDASVNFSEFSKKCSQRWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

Figure 5b continued

> sequence 240 E16H
PDASVNFSEFSKKCSHRWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 241 E16N
PDASVNFSEFSKKCSNRWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 242 R17H
PDASVNFSEFSKKCSEHWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 243 R17Q
PDASVNFSEFSKKCSEQWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 244 W18Y
PDASVNFSEFSKKCSERYKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 245 W18S
PDASVNFSEFSKKCSERSKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 246 K19N
PDASVNFSEFSKKCSERWNTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 247 K19Q
PDASVNFSEFSKKCSERWQTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 248 M21I
PDASVNFSEFSKKCSERWKTISAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 249 M21V
PDASVNFSEFSKKCSERWKTVSAKEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 250 K24N
PDASVNFSEFSKKCSERWKTMSANEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 251 K24Q
PDASVNFSEFSKKCSERWKTMSAQEKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 252 E25Q
PDASVNFSEFSKKCSERWKTMSAKQKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 253 E25H
PDASVNFSEFSKKCSERWKTMSAKHKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 254 E25N
PDASVNFSEFSKKCSERWKTMSAKNKGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 255 K26N
PDASVNFSEFSKKCSERWKTMSAKENGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 256 K26Q
PDASVNFSEFSKKCSERWKTMSAKEQGKFEDMAKADKARYEREMKTYIPPKGET

> sequence 257 K28N
PDASVNFSEFSKKCSERWKTMSAKEKGNFEDMAKADKARYEREMKTYIPPKGET

> sequence 258 K28Q

Figure 5b continued

PDASVNFSEFSKKCSERWKTMSAKEKGQFEDMAKADKARYEREMKTYIPPKGET

> sequence 259 F29I
PDASVNFSEFSKKCSERWKTMSAKEKGKIEDMAKADKARYEREMKTYIPPKGET

> sequence 260 F29V
PDASVNFSEFSKKCSERWKTMSAKEKGKVEDMAKADKARYEREMKTYIPPKGET

> sequence 261 E30Q
PDASVNFSEFSKKCSERWKTMSAKEKGKFQDMAKADKARYEREMKTYIPPKGET

> sequence 262 E30H
PDASVNFSEFSKKCSERWKTMSAKEKGKFHDMAKADKARYEREMKTYIPPKGET

> sequence 263 E30N
PDASVNFSEFSKKCSERWKTMSAKEKGKFNDMAKADKARYEREMKTYIPPKGET

> sequence 264 D31N
PDASVNFSEFSKKCSERWKTMSAKEKGKFENMAKADKARYEREMKTYIPPKGET

> sequence 265 D31Q
PDASVNFSEFSKKCSERWKTMSAKEKGKFEQMAKADKARYEREMKTYIPPKGET

> sequence 266 M32I
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDIAKADKARYEREMKTYIPPKGET

> sequence 267 M32V
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDVAKADKARYEREMKTYIPPKGET

> sequence 268 K34N
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMANADKARYEREMKTYIPPKGET

> sequence 269 K34Q
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAQADKARYEREMKTYIPPKGET

> sequence 270 D36N
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKANKARYEREMKTYIPPKGET

> sequence 271 D36Q
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKAQKARYEREMKTYIPPKGET

> sequence 272 K37N
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADNARYEREMKTYIPPKGET

> sequence 273 K37Q
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADQARYEREMKTYIPPKGET

> sequence 274 R39H
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKAHYEREMKTYIPPKGET

> sequence 275 R39Q
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKAQYEREMKTYIPPKGET

> sequence 276 Y40H
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARHEREMKTYIPPKGET

Figure 5b continued

> sequence 277 Y40I
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARIEREMKTYIPPKGET

> sequence 278 E41Q
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYQREMKTYIPPKGET

> sequence 279 E41H
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYHREMKTYIPPKGET

> sequence 280 E41N
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYNREMKTYIPPKGET

> sequence 281 R42H
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEHEMKTYIPPKGET

> sequence 282 R42Q
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEQEMKTYIPPKGET

> sequence 283 E43Q
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERQMKTYIPPKGET

> sequence 284 E43H
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERHMKTYIPPKGET

> sequence 285 E43N
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYERNMKTYIPPKGET

> sequence 286 M44I
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREIKTYIPPKGET

> sequence 287 M44V
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREVKTYIPPKGET

> sequence 288 K45N
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMNTYIPPKGET

> sequence 289 K45Q
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMQTYIPPKGET

> sequence 290 Y47H
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTHIPPKGET

> sequence 291 Y47I
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTIIPPKGET

> sequence 292 P49A
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIAPKGET

> sequence 293 P49S
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYISPKGET

> sequence 294 P50A
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPAKGET

Figure 5b continued

> sequence 295 P50S
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPSKGET

> sequence 296 K51N
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPNGET

> sequence 297 K51Q
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPQGET

> sequence 298 E53Q
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGQT

> sequence 299 E53H
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGHT

> sequence 300 E53N
PDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIPPKGNT

Figure 6a

Box A 84 amino acid of HMGB1 *Anopheles gambia* (XP_311154)

 # Protection against proteolysis
If sequence:

GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEK
QRFHEMAEKDKARYELEMQSYVPPKGAV

In bold amino acids sensitive to proteases proteolysis

Figure 6b

Box A 84 amino acid
 # Mutant list:

K2N	E24H	F40V	K56N	E71H
K2Q	E24N	R42H	K56Q	E71N
K4N	E25Q	R42Q	R58H	L72I
K4Q	E25H	K43N	R58Q	L72V
D5N	E25N	K43Q	F59I	E73Q
D5Q	K27N	E46Q	F59V	E73H
K7N	K27Q	E46H	E61Q	E73N
K7Q	K28N	E46N	E61H	M74I
P8A	K28Q	R47H	E61N	M74V
P8S	K29N	R47Q	M62I	Y77H
R9H	K29Q	W48Y	M62V	Y77I
R9Q	P31A	W48S	E64Q	P79A
R11H	P31S	K49N	E64H	P79S
R11Q	E32Q	K49Q	E64N	P80A
M12I	E32H	M51I	K65N	P80S
M12V	E32N	M51V	K65Q	K81N
Y15H	E33Q	L52I	D66N	K81Q
Y15I	E33H	L52V	D66Q	
F17I	E33N	D53N	K67N	
F17V	F37I	D53Q	K67Q	
F18I	F37V	K54N	R69H	
F18V	E39Q	K54Q	R69Q	
R23H	E39H	E55Q	Y70H	
R23Q	E39N	E55H	Y70I	
E24Q	F40I	E55N	E71Q	

Figure 6b continued

> SEQUENCE 301 Wild type
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 302 K2N
GNVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 303 K2Q
GQVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 304 K4N
GKVNDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 305 K4Q
GKVQDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 306 D5N
GKVKNNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 307 D5Q
GKVKQNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 308 K7N
GKVKDNNPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 309 K7Q
GKVKDNQPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 310 P8A
GKVKDNKARGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 311 P8S
GKVKDNKSRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 312 R9H
GKVKDNKPHGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 313 R9Q
GKVKDNKPQGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 314 R11H
GKVKDNKPRGHMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK

Figure 6b continued

ARYELEMQSYVPPKGAV

>> SEQUENCE 315 R11Q
GKVKDNKPRGQMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 316 M12I
GKVKDNKPRGRITAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKA
RYELEMQSYVPPKGAV

>> SEQUENCE 317 M12V
GKVKDNKPRGRVTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 318 Y15H
GKVKDNKPRGRMTAHAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 319 Y15I
GKVKDNKPRGRMTAIAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKA
RYELEMQSYVPPKGAV

>> SEQUENCE 320 F17I
GKVKDNKPRGRMTAYAIFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKA
RYELEMQSYVPPKGAV

>> SEQUENCE 321 F17V
GKVKDNKPRGRMTAYAVFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 322 F18I
GKVKDNKPRGRMTAYAFIVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKA
RYELEMQSYVPPKGAV

>> SEQUENCE 323 F18V
GKVKDNKPRGRMTAYAFVVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 324 R23H
GKVKDNKPRGRMTAYAFFVQTCHEEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 325 R23Q
GKVKDNKPRGRMTAYAFFVQTCQEEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 326 E24Q
GKVKDNKPRGRMTAYAFFVQTCRQEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 327 E24H
GKVKDNKPRGRMTAYAFFVQTCRHEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 328 E24N

Figure 6b continued

GKVKDNKPRGRMTAYAFFVQTCRNEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 329 E25Q
GKVKDNKPRGRMTAYAFFVQTCREQHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 330 E25H
GKVKDNKPRGRMTAYAFFVQTCREHHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 331 E25N
GKVKDNKPRGRMTAYAFFVQTCRENHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 332 K27N
GKVKDNKPRGRMTAYAFFVQTCREEHNKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 333 K27Q
GKVKDNKPRGRMTAYAFFVQTCREEHQKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 334 K28N
GKVKDNKPRGRMTAYAFFVQTCREEHKNKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 335 K28Q
GKVKDNKPRGRMTAYAFFVQTCREEHKQKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 336 K29N
GKVKDNKPRGRMTAYAFFVQTCREEHKKNHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 337 K29Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKQHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 338 P31A
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHAEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 339 P31S
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHSEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 340 E32Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPQEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 341 E32H
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPHEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK

Figure 6b continued

ARYELEMQSYVPPKGAV

>> SEQUENCE 342 E32N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPNEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 343 E33Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEQQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 344 E33H
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEHQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 345 E33N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPENQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 346 F37I
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIIAEFSRKCAERWKTMLDKEKQRFHEMAEKDKA
RYELEMQSYVPPKGAV

> > SEQUENCE 347 F37V
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIVAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 348 E39Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAQFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 349 E39H
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAHFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 350 E39N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFANFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 351 F40I
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEISRKCAERWKTMLDKEKQRFHEMAEKDKA
RYELEMQSYVPPKGAV

> > SEQUENCE 352 F40V
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEVSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 353 R42H
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSHKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 354 R42Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSQKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 355 K43N

Figure 6b continued

GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRNCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 356 K43Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRQCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 357 E46Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAQRWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 358 E46H
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAHRWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 359 E46N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCANRWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 360 R47H
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAEHWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 361 R47Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAEQWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 362 W48Y
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERYKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 363 W48S
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERSKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 364 K49N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWNTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 365 K49Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWQTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 366 M51I
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTILDKEKQRFHEMAEKDKA
RYELEMQSYVPPKGAV

> > SEQUENCE 367 M51V
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTVLDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > > SEQUENCE 368 L52I
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMIDKEKQRFHEMAEKDKA
RYELEMQSYVPPKGAV

Figure 6b continued

> > SEQUENCE 369 L52V
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMVDKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 370 D53N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLNKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 371 D53Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLQKEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 372 K54N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDNEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 373 K54Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDQEKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 374 E55Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKQKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 375 E55H
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKHKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 376 E55N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKNKQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 377 K56N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKENQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 378 K56Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEQQRFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 379 R58H
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQHFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 380 R58Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQQFHEMAEKDK
ARYELEMQSYVPPKGAV

> > SEQUENCE 381 F59I
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRIHEMAEKDKA
RYELEMQSYVPPKGAV

> > SEQUENCE 382 F59V
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRVHEMAEKDK
ARYELEMQSYVPPKGAV

Figure 6b continued

>> SEQUENCE 383 E61Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHQMAEKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 384 E61H
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHHMAEKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 385 E61N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHNMAEKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 386 M62I
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEIAEKDKA
RYELEMQSYVPPKGAV

>> SEQUENCE 387 M62V
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEVAEKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 388 E64Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAQKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 389 E64H
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAHKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 390 E64N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMANKDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 391 K65N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAENDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 392 K65Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEQDK
ARYELEMQSYVPPKGAV

>> SEQUENCE 393 D66N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKNK
ARYELEMQSYVPPKGAV

>> SEQUENCE 394 D66Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKQK
ARYELEMQSYVPPKGAV

>> SEQUENCE 395 K67N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDN
ARYELEMQSYVPPKGAV

>> SEQUENCE 396 K67Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDQ

Figure 6b continued

ARYELEMQSYVPPKGAV

>> SEQUENCE 397 R69H
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
AHYELEMQSYVPPKGAV

>> SEQUENCE 398 R69Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
AQYELEMQSYVPPKGAV

>> SEQUENCE 399 Y70H
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARHELEMQSYVPPKGAV

>> SEQUENCE 400 Y70I
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARIELEMQSYV

>> SEQUENCE 401 E71Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYQLEMQSYVPPKGAV

>> SEQUENCE 402 E71H
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYHLEMQSYVPPKGAV

>> SEQUENCE 403 E71N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYNLEMQSYVPPKGAV

>> SEQUENCE 404 L72I
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYEIEMQSYVPPKGAV

>> SEQUENCE 405 L72V
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYEVEMQSYVPPKGAV

>> SEQUENCE 406 E73Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELQMQSYVPPKGAV

>> SEQUENCE 407 E73H
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELHMQSYVPPKGAV

>> SEQUENCE 408 E73N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELNMQSYVPPKGAV

>> SEQUENCE 409 M74I
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEIQSYVPPKGAV

>> SEQUENCE 410 M74V
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK

Figure 6b continued

ARYELEVQSYVPPKGAV

>> SEQUENCE 411 Y77H
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSHVPPKGAV

>> SEQUENCE 412 Y77I
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSIVPPKGAV

>> SEQUENCE 413 P79A
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVAPKGAV

>> SEQUENCE 414 P79S
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVSPKGAV

>> SEQUENCE 415 P80A
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPAKGAV

>> SEQUENCE 416 P80S
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPSKGAV

>> SEQUENCE417 K81N
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPNGAV

>> SEQUENCE 418 K81Q
GKVKDNKPRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDK
ARYELEMQSYVPPQGAV

Figure 7a

Box A 77 amino acid of HMGB1 *Anopheles gambia* (XP_311154)

 # Protection against proteolysis
If sequence:

PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMA
EKDKARYELEMQSYVPPKGAV

In bold amino acids sensitive to proteases proteolysis

Figure 7b

Box A 77 amino acid of HMGB1 *Anopheles gambia* (XP_311154)

 # Mutant list:

P1A	E26N	R51Q	P73A
P1S	F30I	F52I	P73S
R2H	F30V	F52V	K74N
R2Q	E32Q	E54Q	K74Q
R4H	E32H	E54H	
R4Q	E32N	E54N	
M5I	F33I	M55I	
M5V	F33V	M55V	
Y8H	R35H	E57Q	
Y8I	R35Q	E57H	
F10I	K36N	E57N	
F10V	K36Q	K58N	
F11I	E39Q	K58Q	
F11V	E39H	D59N	
R16H	E39N	D59Q	
R16Q	R40H	K60N	
E17Q	R40Q	K60Q	
E17H	W41Y	R62H	
E17N	W41S	R62Q	
E18Q	K42N	Y63H	
E18H	K42Q	Y63I	
E18N	M44I	E64Q	
K20N	M44V	E64H	
K20Q	L45I	E64N	
K21N	L45V	L65I	
K21Q	D46N	L65V	
K22N	D46Q	E66Q	
K22Q	K47N	E66H	
P24A	K47Q	E66N	
P24S	E48Q	M67I	
E25Q	E48H	M67V	
E25H	E48N	Y70H	
E25N	K49N	Y70I	
E26Q	K49Q	P72A	
E26H	R51H	P72S	

Figure 7b continued

> SEQUENCE 419 Wild type
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> > SEQUENCE 420 P1A
ARGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 421 P1S
SRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 422 R2H
PHGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 423 R2Q
PQGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 424 R4H
PRGHMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 425 R4Q
PRGQMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 426 M5I
PRGRITAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQ
SYVPPKGAV

> SEQUENCE 427 M5V
PRGRVTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 428 Y8H
PRGRMTAHAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 429 Y8I
PRGRMTAIAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQ
SYVPPKGAV

> SEQUENCE 430 F10I
PRGRMTAYAIFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQ
SYVPPKGAV

> SEQUENCE 431 F10V
PRGRMTAYAVFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

Figure 7b continued

> SEQUENCE 432 F11I
PRGRMTAYAFIVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQ
SYVPPKGAV

> SEQUENCE 433 F11V
PRGRMTAYAFVVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 434 R16H
PRGRMTAYAFFVQTCHEEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 435 R16Q
PRGRMTAYAFFVQTCQEEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 436 E17Q
PRGRMTAYAFFVQTCRQEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 437 E17H
PRGRMTAYAFFVQTCRHEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 438 E17N
PRGRMTAYAFFVQTCRNEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 439 E18Q
PRGRMTAYAFFVQTCREQHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 440 E18H
PRGRMTAYAFFVQTCREHHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 441 E18N
PRGRMTAYAFFVQTCRENHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 442 K20N
PRGRMTAYAFFVQTCREEHNKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 443 K20Q
PRGRMTAYAFFVQTCREEHQKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 444 K21N
PRGRMTAYAFFVQTCREEHKNKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 445 K21Q
PRGRMTAYAFFVQTCREEHKQKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

Figure 7b continued

> SEQUENCE 446 K22N
PRGRMTAYAFFVQTCREEHKKNHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 447 K22Q
PRGRMTAYAFFVQTCREEHKKQHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 448 P24A
PRGRMTAYAFFVQTCREEHKKKHAEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 449 P24S
PRGRMTAYAFFVQTCREEHKKKHSEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 450 E25Q
PRGRMTAYAFFVQTCREEHKKKHPQEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 451 E25H
PRGRMTAYAFFVQTCREEHKKKHPHEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 452 E25N
PRGRMTAYAFFVQTCREEHKKKHPNEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 453 E26Q
PRGRMTAYAFFVQTCREEHKKKHPEQQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 454 E26H
PRGRMTAYAFFVQTCREEHKKKHPEHQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 455 E26N
PRGRMTAYAFFVQTCREEHKKKHPENQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 456 F30I
PRGRMTAYAFFVQTCREEHKKKHPEEQVIIAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQ
SYVPPKGAV

> SEQUENCE 457 F30V
PRGRMTAYAFFVQTCREEHKKKHPEEQVIVAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 458 E32Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAQFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 459 E32H
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAHFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

Figure 7b continued

> SEQUENCE 460 E32N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFANFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 461 F33I
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEISRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQ
SYVPPKGAV

> SEQUENCE 462 F33V
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEVSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 463 R35H
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSHKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 464 R35Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSQKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 465 K36N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRNCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 466 K36Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRQCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 467 E39Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAQRWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 468 E39H
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAHRWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 469 E39N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCANRWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 470 R40H
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAEHWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 471 R40Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAEQWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 472 W41Y
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERYKTMLDKEKQRFHEMAEKDKARYELEMQ
SYVPPKGAV

> SEQUENCE 473 W41S

Figure 7b continued

PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERSKTMLDKEKQRFHEMAEKDKARYELEMQ
SYVPPKGAV

> SEQUENCE 474 K42N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWNTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 475 K42Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWQTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 476 M44I
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTILDKEKQRFHEMAEKDKARYELEMQ
SYVPPKGAV

> SEQUENCE 477M44V
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTVLDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 478 L45I
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMIDKEKQRFHEMAEKDKARYELEMQ
SYVPPKGAV

> SEQUENCE 479 L45V
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMVDKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 480 D46N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLNKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 481 D46Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLQKEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 482 K47N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDNEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 483 K47Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDQEKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 484 E48Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKQKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 485 E48H
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKHKQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 486 E48N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKNKQRFHEMAEKDKARYELEM
QSYVPPKGAV

Figure 7b continued

> SEQUENCE 487 K49N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKENQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 488 K49Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEQQRFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 489 R51H
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQHFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 490 R51Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQQFHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 491 F52I
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRIHEMAEKDKARYELEMQ
SYVPPKGAV

> SEQUENCE 492 F52V
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRVHEMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 493 E54Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHQMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 494 E54H
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHHMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 495 E54N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHNMAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 496 M55I
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEIAEKDKARYELEMQ
SYVPPKGAV

> SEQUENCE 497 M55V
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEVAEKDKARYELEM
QSYVPPKGAV

> SEQUENCE 498 E57Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAQKDKARYELEM
QSYVPPKGAV

> SEQUENCE 499 E57H
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAHKDKARYELEM
QSYVPPKGAV

> SEQUENCE 500 E57N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMANKDKARYELEM
QSYVPPKGAV

Figure 7b continued

> SEQUENCE 501 K58N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAENDKARYELEM
QSYVPPKGAV

> SEQUENCE 502 K58Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEQDKARYELEM
QSYVPPKGAV

> SEQUENCE 503 D59N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKNKARYELEM
QSYVPPKGAV

> SEQUENCE 504 D59Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKQKARYELEM
QSYVPPKGAV

> SEQUENCE 505 K60N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDNARYELEM
QSYVPPKGAV

> SEQUENCE 506 K60Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDQARYELEM
QSYVPPKGAV

> SEQUENCE 507 R62H
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKAHYELEM
QSYVPPKGAV

> SEQUENCE 508 R62Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKAQYELEM
QSYVPPKGAV

> SEQUENCE 509 Y63H
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARHELEM
QSYVPPKGAV

> SEQUENCE 510 Y63I
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARIELEMQ
SYVPPKGAV

> SEQUENCE 511 E64Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYQLEM
QSYVPPKGAV

> SEQUENCE 512 E64H
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYHLEM
QSYVPPKGAV

> SEQUENCE 513 E64N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYNLEM
QSYVPPKGAV

> SEQUENCE 514 L65I
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYEIEMQ

Figure 7b continued

SYVPPKGAV

> SEQUENCE 515 L65V
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYEVEM
QSYVPPKGAV

> SEQUENCE 516 E66Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELQM
QSYVPPKGAV

> SEQUENCE 517 E66H
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELHM
QSYVPPKGAV

> SEQUENCE 518 E66N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELNM
QSYVPPKGAV

> SEQUENCE 519 M67I
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEIQ
SYVPPKGAV

> SEQUENCE 520 M67V
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEV
QSYVPPKGAV

> SEQUENCE 521 Y70H
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSHVPPKGAV

> SEQUENCE 523 Y70I
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSIVPPKGAV

> SEQUENCE 524 P72A
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVAPKGAV

> SEQUENCE 525 P72S
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVSPKGAV

> SEQUENCE 526 P73A
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPAKGAV

> SEQUENCE 527 P73S
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPSKGAV

> SEQUENCE 528 K74N
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM
QSYVPPNGAV

> SEQUENCE 529 K74Q
PRGRMTAYAFFVQTCREEHKKKHPEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEM

Figure 7b continued

QSYVPPQGAV

Figure 8a

Box A 54 amino acid of HMGB1 *Anopheles gambia* (XP_311154).

 # Protection against proteolysis
If sequence:

PEEQVIFAE**FSRK**CAE**RWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPP**KGAV

In bold amino acids sensitive to proteases proteolysis

Figure 8b

Box A 54 amino acid of HMGB1 *Anopheles gambia* (XP_311154)

 # Mutant list:

P1A	K24N	E43Q
P1S	K24Q	E43H
E2Q	E25Q	E43N
E2H	E25H	M44I
E2N	E25N	M44V
E3Q	K26N	Y47H
E3H	K26Q	Y47I
E3N	R28H	P49A
F7I	R28Q	P49S
F7V	F29I	P50A
E9Q	F29V	P50S
E9H	E31Q	K51N
E9N	E31H	K51Q
F10I	E31N	
F10V	M32I	
R12H	M32V	
R12Q	E34Q	
K13N	E34H	
K13Q	E34N	
E16Q	K35N	
E16H	K35Q	
E16N	D36N	
R17H	D36Q	
R17Q	K37N	
W18Y	K37Q	
W18S	R39H	
K19N	R39Q	
K19Q	Y40H	
M21I	Y40I	
M21V	E41Q	
L22I	E41H	
L22V	E41N	
D23N	L42I	
D23Q	L42V	

Figure 8b continued

> SEQUENCE 530 Wild type

PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 531 P1A
AEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 532 P1S
SEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 533 E2Q
PQEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 534 E2H
PHEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 535 E2N
PNEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 536 E3Q
PEQQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 537 E3H
PEHQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 538 E3N
PENQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 539 F7I
PEEQVIIAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 540 F7V
PEEQVIVAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 541 E9Q
PEEQVIFAQFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 542 E9H
PEEQVIFAHFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 543 E9N
PEEQVIFANFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 544 F10I
PEEQVIFAEISRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 545 F10V
PEEQVIFAEVSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 546 R12H
PEEQVIFAEFSHKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 547 R12Q
PEEQVIFAEFSQKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

Figure 8b continued

> SEQUENCE 548 K13N
PEEQVIFAEFSRNCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 549 K13Q
PEEQVIFAEFSRQCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 550 E16Q
PEEQVIFAEFSRKCAQRWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 551 E16H
PEEQVIFAEFSRKCAHRWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 552 E16N
PEEQVIFAEFSRKCANRWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 553 R17H
PEEQVIFAEFSRKCAEHWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 554 R17Q
PEEQVIFAEFSRKCAEQWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 555 W18Y
PEEQVIFAEFSRKCAERYKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 556 W18S
PEEQVIFAEFSRKCAERSKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 557 K19N
PEEQVIFAEFSRKCAERWNTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 558 K19Q
PEEQVIFAEFSRKCAERWQTMLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 559 M21I
PEEQVIFAEFSRKCAERWKTILDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 560 M21V
PEEQVIFAEFSRKCAERWKTVLDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 561 L22I
PEEQVIFAEFSRKCAERWKTMIDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 562 L22V
PEEQVIFAEFSRKCAERWKTMVDKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 563 D23N
PEEQVIFAEFSRKCAERWKTMLNKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 564 D23Q
PEEQVIFAEFSRKCAERWKTMLQKEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 565 K24N
PEEQVIFAEFSRKCAERWKTMLDNEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 566 K24Q
PEEQVIFAEFSRKCAERWKTMLDQEKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 567 E25Q

Figure 8b continued

PEEQVIFAEFSRKCAERWKTMLDKQKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 568 E25H
PEEQVIFAEFSRKCAERWKTMLDKHKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 569 E25N
PEEQVIFAEFSRKCAERWKTMLDKNKQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 570 K26N
PEEQVIFAEFSRKCAERWKTMLDKENQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 571 K26Q
PEEQVIFAEFSRKCAERWKTMLDKEQQRFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 572 R28H
PEEQVIFAEFSRKCAERWKTMLDKEKQHFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 573 R28Q
PEEQVIFAEFSRKCAERWKTMLDKEKQQFHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 574 F29I
PEEQVIFAEFSRKCAERWKTMLDKEKQRIHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 575 F29V
PEEQVIFAEFSRKCAERWKTMLDKEKQRVHEMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 576 E31Q
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHQMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 577 E31H
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHHMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 578 E31N
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHNMAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 579 M32I
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEIAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 580 M32V
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEVAEKDKARYELEMQSYVPPKGAV

> SEQUENCE 581 E34Q
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAQKDKARYELEMQSYVPPKGAV

> SEQUENCE 582 E34H
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAHKDKARYELEMQSYVPPKGAV

> SEQUENCE 583 E34N
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMANKDKARYELEMQSYVPPKGAV

> SEQUENCE 584 K35N
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAENDKARYELEMQSYVPPKGAV

> SEQUENCE 585 K35Q
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEQDKARYELEMQSYVPPKGAV

> SEQUENCE 586 D36N

Figure 8b continued

PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKNKARYELEMQSYVPPKGAV

> SEQUENCE 587 D36Q
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKQKARYELEMQSYVPPKGAV

> SEQUENCE 588 K37N
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDNARYELEMQSYVPPKGAV

> SEQUENCE 590 K37Q
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDQARYELEMQSYVPPKGAV

> SEQUENCE 591 R39H
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKAHYELEMQSYVPPKGAV

> SEQUENCE 592 R39Q
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKAQYELEMQSYVPPKGAV

> SEQUENCE 593 Y40H
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARHELEMQSYVPPKGAV

> SEQUENCE 594 Y40I
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARIELEMQSYVPPKGAV

> SEQUENCE 595 E41Q
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYQLEMQSYVPPKGAV

> SEQUENCE 596 E41H
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYHLEMQSYVPPKGAV

> SEQUENCE 597 E41N
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYNLEMQSYVPPKGAV

> SEQUENCE 598 L42I
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYEIEMQSYVPPKGAV

> SEQUENCE 599 L42V
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYEVEMQSYVPPKGAV

> SEQUENCE 600 E43Q
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELQMQSYVPPKGAV

> SEQUENCE 601 E43H
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELHMQSYVPPKGAV

> SEQUENCE 602 E43N
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELNMQSYVPPKGAV

> SEQUENCE 603 M44I
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEIQSYVPPKGAV

> SEQUENCE 604 M44V
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEVQSYVPPKGAV

> SEQUENCE 605 Y47H
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSHVPPKGAV

> SEQUENCE 606 Y47I
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSIVPPKGAV

Figure 8b continued

> SEQUENCE 607 P49A
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVAPKGAV

> SEQUENCE 608 P49S
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVSPKGAV

> SEQUENCE 609 P50A
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPAKGAV

> SEQUENCE 610 P50S
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPSKGAV

> SEQUENCE 611 K51N
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPNGAV

> SEQUENCE 612 K51Q
PEEQVIFAEFSRKCAERWKTMLDKEKQRFHEMAEKDKARYELEMQSYVPPQGAV

Figure 9

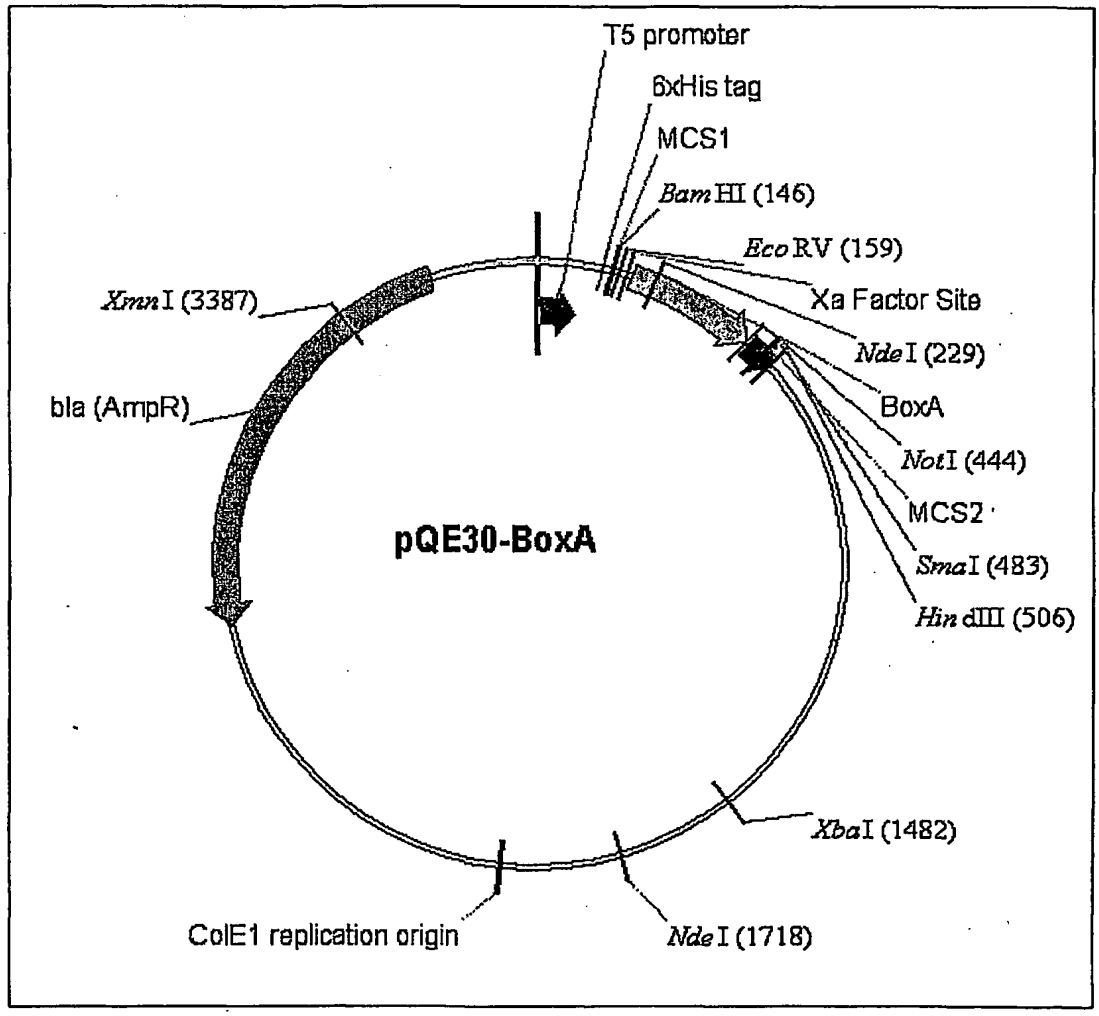

T5 promoter
6xHis tag
MCS1
BamHI (146)
EcoRV (159)
Xa Factor Site
NdeI (229)
BoxA
NotI (444)
MCS2
SmaI (483)
HindIII (506)
XbaI (1482)
NdeI (1718)
ColE1 replication origin
bla (AmpR)
XmnI (3387)
pQE30-BoxA

Figure 10

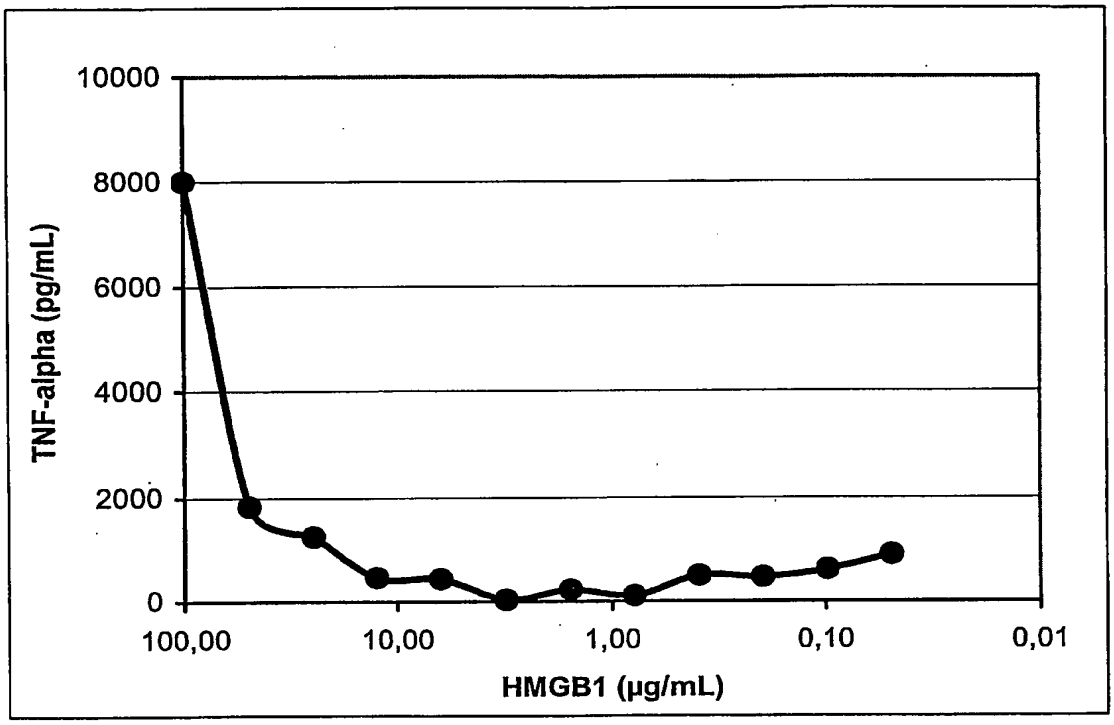
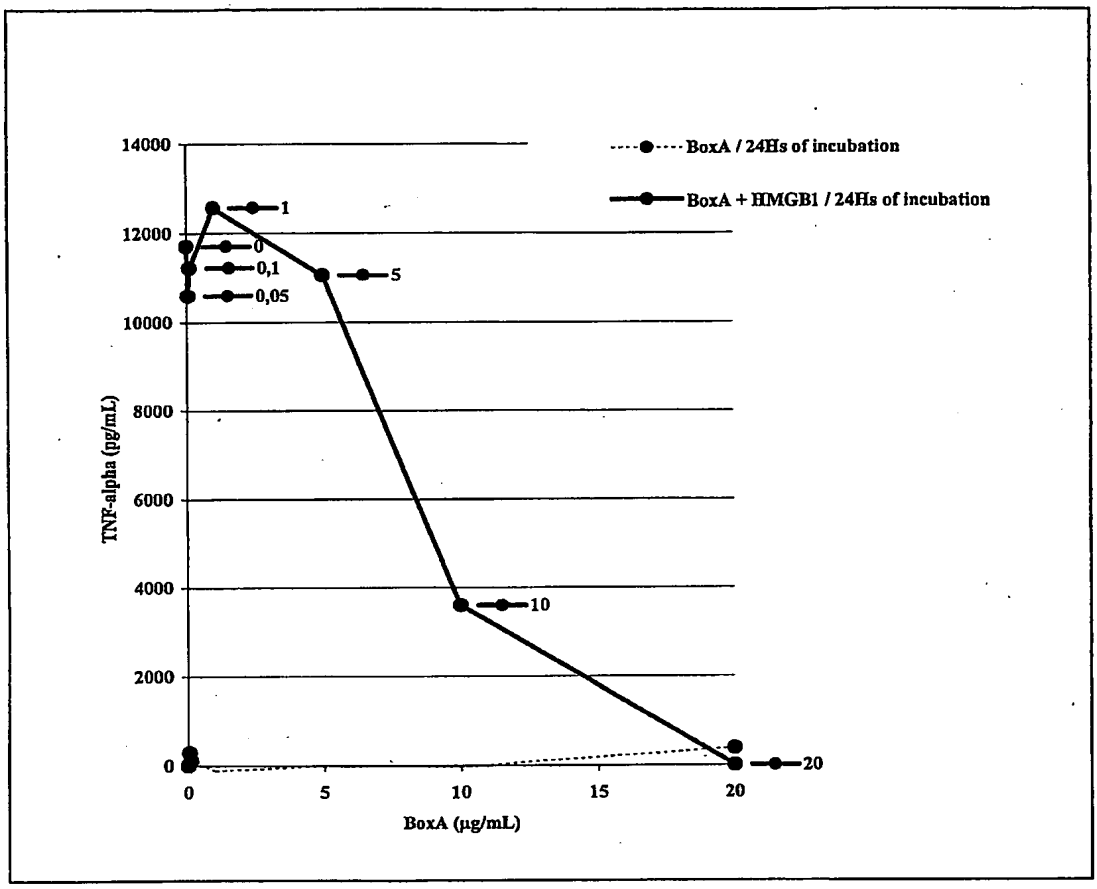

10000
8000
6000
4000
2000
0
TNF-alpha (pg/mL)
100,00
10,00
1,00
0,10
0,01
HMGB1 (µg/mL)

Figure 11


BoxA / 24Hs of incubation
BoxA + HMGB1 / 24Hs of incubation
TNF-alpha (pg/mL)
BoxA (µg/mL)
14000
12000
10000
8000
6000
4000
2000
0
0
5
10
15
20
1
0
0,1
0,05
5
10
20

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(74) Agent: **Weickmann & Weickmann**; Postfach 860 820, 81635 München (DE).

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Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
1 June 2006

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PROTEASE RESISTANT HUMAN AND NON-HUMAN HMGB1 BOX-A MUTANTS AND THEIR THERAPEUTIC/DIAGNOSTIC USE

(57) Abstract: The present invention relates to polypeptide variants of the HMGB-1 high affinity binding domain Box-A (HMGB1 Box-A) or to a biologically active fragment of HMGB1 Box-A, which are obtained through systematic mutations of single amino acids of the wild-type HMGB1 Box-A protein and which show an increased resistance to proteases and which are therefore characterized by more favourable pharmacokinetic and pharmacodynamic profiles. Moreover, the present invention concerns the use of said polypeptide molecules of HMGB1 Box-A to diagnose, prevent, alleviate and/or treat pathologies associated with extracellular HMGB1.

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2005/009528

A. CLASSIFICATION OF SUBJECT MATTER
C07K14/47 C12N15/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, Sequence Search, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/046345 A (CRITICAL THERAPEUTICS, INC; NEWMAN, WALTER; O'KEEFE, THERESA, L) 3 June 2004 (2004-06-03) the whole document claims 15,20,37,44 page 40, paragraph 2 - page 42, last paragraph	1-5, 13-28, 33,34, 36,37
X	FARID RAMY S ET AL: "Differential binding of HMG1, HMG2, and a single HMG box to cisplatin-damaged DNA" TOXICOLOGY AND APPLIED PHARMACOLOGY, vol. 141, no. 2, 1996, pages 532-539, XP002368580 ISSN: 0041-008X the whole document figure 1	1-5,7-9, 18

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2006	03/04/2006

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lechner, O

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2005/009528

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FALCIOLA LUCA ET AL: "Mutational analysis of the DNA binding domain A of chromosomal protein HMG1" NUCLEIC ACIDS RESEARCH, vol. 22, no. 3, 1994, pages 285-292, XP002368581 ISSN: 0305-1048 the whole document figure 1 page 291, paragraph 2	1-5,7-9, 13-20
X	WO 02/092004 A (NORTH SHORE-LONG ISLAND JEWISH RESEARCH INSTITUTE; THE GENERAL HOSPITA) 21 November 2002 (2002-11-21)	1-5,7, 13-20, 22-25, 33,36,37
Y	the whole document page 32, lines 15-30 page 20, line 22 - page 22, line 12 page 36, lines 17-19	1-41
Y	WO 2004/022747 A (NAUTILUS BIOTECH; GANTIER, RENE; GUYON, THIERRY; CRUZ RAMOS, HUGO; VEG) 18 March 2004 (2004-03-18) cited in the application abstract page 125; example 2 page 41, last paragraph - page 42, paragraph 1	1-41
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2005/009528

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANDERSSON U ET AL: "HMGB1 is a potent trigger of arthritis." JOURNAL OF INTERNAL MEDICINE, vol. 255, no. 3, March 2004 (2004-03), pages 344-350, XP002368583 ISSN: 0954-6820 cited in the application the whole document	1-41
Y	PARK JONG SUNG ET AL: "Involvement of Toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 279, no. 9, 27 February 2004 (2004-02-27), pages 7370-7377, XP002368584 ISSN: 0021-9258 abstract	29
A	YANG HUAN ET AL: "Reversing established sepsis with antagonists of endogenous high-mobility group box 1." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 101, no. 1, 6 January 2004 (2004-01-06), pages 296-301, XP002368586 ISSN: 0027-8424 cited in the application . the whole document	
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2005/009528

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	CH 694 905 A5 (MARCO OSTINI) 15 September 2005 (2005-09-15) the whole document -----	1,3-6, 18, 22-25, 33,36,37
P,A	WO 2005/025604 A (THE GENERAL HOSPITAL CORPORATION; NORTH SHORE-LONG ISLAND JEWISH RESEA) 24 March 2005 (2005-03-24) the whole document -----	1-41

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2005/009528

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claim 37 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claim 37 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box II.2

Claims 1-6, 13-41 are unclear in the sense of Art. 6, PCT as far as relating to to an extremely large number of possible variants.
1) From the present wording it is unclear whether HMGB1 (= amphoterin = HMG1 = HMG3) fragments from Anopheles gambia have to be considered as variant of the human HMGB1 and vice versa.
2) Said claims (with the exception of claim 2) are also unclear (Art. 6, PCT) since the amount of mutations etc. is not limited, i.e. any protein sequence would appear to fall under the definition of e.g. present claim 1.
Consequently, the search was restricted to those variant polypeptides which appear to be clear, i.e. HMGB1 A-box polypeptides or biologically active fragments thereof carrying 1-10 mutations by substitution, deletion or an addition of single amino acids (c.f. claims 2-3).

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2005/009528

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO 2005025604 A	24-03-2005	NONE	